A Study of Host Factors Influencing Retroviral Infectivity

Zuzana Keckesova

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Wohl Virion Centre
Department of Immunology and Molecular Pathology
Windeyer Institute of Medical Sciences
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I, Zuzana Keckesova, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

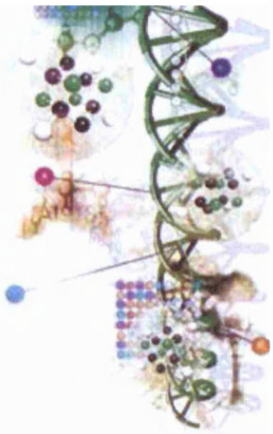
While mammalian cells provide many of the molecules and machinery required by retroviruses to successfully achieve their replication cycle, they have also developed specific defences to protect themselves against viral infection. Existence of these antiviral "restriction" factors accounts for the limited ability of viruses to spread from one species to another. If a new virus becomes successfully established in its new host then it can cause serious disease, as is the case in the HIV pandemic. It is thus important to identify and understand how these mammalian protective factors work, to understand the molecular mechanisms involved and how viruses can evade them.

The aim of my PhD thesis is to better understand the role of these restriction factors in innate immunity. My work in the last 3 years has been to examine various aspects of retroviral restriction by a cellular protein called TRIM5alpha.

The first part of my PhD thesis shows that the Lv1 and Ref1 class of restriction factors are species-specific variants of TRIM5α, a factor that was shown to block HIV-1 infection in Rhesus macaque cells. Differential splicing of TRIM5 transcripts results in the production of several isoforms that lack the TRIM5α C-terminal B30.2 domain. To examine the contribution of these short TRIM5 splice variants to antiviral activity I cloned the TRIM5δ isoform. I have shown that expression of human TRIM5δ in cat and human cells had a dominant negative effect on the activity of co-expressed human TRIM5α.

The second part of my PhD thesis focuses on the role of the peptidyl prolyl isomerase enzyme cyclophilin A in TRIM5α's antiviral activity. It shows that cyclophilin A renders HIV-1 sensitive to restriction by TRIM5α in cells from Old World monkeys and that in human cells the effects of cyclophilin A on HIV-1 infection are independent of TRIM5α.

The third part of my thesis investigates the effects of arsenic trioxide, a drug that modifies the behaviour of the TRIM protein PML (TRIM19), on TRIM5α mediated restriction. By using molecular biology, biochemistry and microscopy I showed that arsenic influences retroviral replication through its effect on TRIM5α and that it causes the degradation of this restriction factor.



For my parents

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Abbreviations

Agm	African green monkey
AID	Activation-induced deaminase
AIDS	Acquired immunodeficiency syndrome
AIP1/Alix	ALG-2 interacting protein 1
AMP	Adenosine monophosphate
AP-1	Activating protein-1 transcription factor
AP2	Adaptor protein complex 2
APL	Acute promyelocytic leukaemia
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide
Arp2/3	Actin-Related Proteins 2/3
As_2O_3	Arsenic trioxide
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAF	Barrier-to-autointegration factor
Bcl-2	B-cell lymphoma–leukaemia gene 2
B-MLV	B-tropic murine leukaemia virus
bp	Base-pair
CA	Capsid protein
CA^N	N-terminal domain of capsid protein
CCR5	Chemokine (C-C motif) receptor 5
CD	Cluster of differentiation
CDC	Centres for Disease Control
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CHMP	Charged multivesicular body protein
CO_2	Carbon dioxide
cppt	Central copy of the polypurine tract region
CPSF6	Cleavage and polyadenylation specific factor 6
Crm1	Chromosome region maintenance 1
CSA	Cyclosporine A
CUL5	Cullin-5
CXCR4	Chemokine receptor 4
CypA	Cyclophilin A
CypB	Cyclophilin B
dATP	Deoxyadenosine triphosphate
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DC-SIGNR	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin-related
DDX3	DEAD box RNA helicase member 3
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate mix
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
Env	Envelope protein products
ERV-L	Endogenous retrovirus group L
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FIV	Feline immunodeficiency virus
FKBP	FK506 binding protein
FL	Fluorescence
FMF	Familial mediterranean fever

Fv1	Friend virus susceptibility gene 1
Fv4	Friend virus susceptibility gene 4
fwd	Forward
Gag	Group associated antigen
GALT	Gut-associated lymphoid tissue
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
Gly	Glycine
gp	Glycoprotein
GRID	Gay-related immunodeficiency disease
GTP	Guanosine triphosphate
HAART	Highly active anti-retroviral therapy
HA-tag	Haemagglutinin tag
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HIV-1	Human immunodeficiency virus type-1
HIV-2	Human immunodeficiency virus type-2
HMG A1	High mobility group chromosomal protein A1
hPOSH	Human-plenty Of SH3 domains
HRP	Horseradish peroxidase
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
Hsp90	Heat shock protein 90
HSV-1	Herpes simplex virus-1
HTLV	Human T-cell leukaemia virus or human T lymphotropic virus
hu	human
I-domain	Interaction domain
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IN	Integrase
Ini1	Integrase interactor 1
ISRE	Interferon-stimulated response element
Itk	Interleukin-2 tyrosine kinase
Kb	Kilobases
KD	Knock-down
kDa	Kilodaltons
Kif4	Kinesin super family protein member 4
LacZ	Bacterial beta -galactosidase gene
LAS	Lymphadenopathy syndrome
LAV	Lymphadenopathy-associated virus
LB-agar	Luria broth mixed with agar
LC8	Light chain 8
L-domain	Late domain
LEDGF	Lens epithelium-derived growth factor
LINE-1	Long interspersed nucleotide element-1
LTR	Long terminal repeat
Lv1	Lentivirus susceptibility factor 1
Lv2	Lentivirus susceptibility factor 2
MA	Matrix protein
MAPK	Mitogen-activated protein kinase
M-domain	Membrane targeting domain
M-group	Main group of HIV-1
Mg_2SO_4	Magnesium sulphate
MHC	Major histocompatibility complex
MHR	Major homology region
MID1	Midline defect protein 1
MLV	Murine leukaemia virus
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MTOC	Microtubule organizing centre
MoMLV	Moloney murine leukaemia virus

MVB	Multivesicular body
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB-MLV	NB-tropic murine leukaemia virus
NC	Nucleocapsid protein
Nef	Negative factor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor of κ light polypeptide gene enhancer in B-cells
N-group	Non M/non O group of HIV-1
NLS	Nuclear localization signal
N-MLV	N-tropic murine leukaemia virus
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NPCs	Nuclear pore complexes
NRTIs	Nucleoside/nucleotide reverse transcriptase inhibitors
O-group	Outlier group of HIV-1
oligo	Oligonucleotide
OMK	Owl monkey cells
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
pbs	Primer binding site
pBS	Plasmid Bluescript S-K polylinker direction
PBS	Phosphate-buffered saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PICs	Pre-integration complexes
PIs	Protease inhibitors
PML	Promyelocytic leukaemia protein
pol	Polymerase-encoding polyprotein
PPIase	Peptidyl prolyl isomerase
ppt	Polypurine tract
PR, pro	Protease
Pro	Proline
puro	Puromycin resistance gene
PVDF	Polyvinylidene fluoride
Ran	Ras-related protein
RARα	Retinoic acid receptor alpha
RBCC	RING-B-Box-Coiled-Coil motif
RBX1	Ring-box-1
Ref1	Restriction factor 1
Rev	Regulator of viral gene expression
RFP	Red fluorescent protein
RING	Really interesting new gene
RIP	Rev interacting protein
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rpm	Revolutions per minute
RRE	Rev Response Element
RT	Reverse transcriptase
RTCs	Reverse transcription complexes
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
SIV	Simian immunodeficiency virus
SIV_{Agm}	Simian immunodeficiency virus from African green monkey
SIV_{cpz}	Simian immunodeficiency virus from Chimpanzee
SIV_{gor}	Simian immunodeficiency virus from Gorilla
SIV_{gsn}	Simian immunodeficiency virus from Greater spot-nosed monkey
SIV_{mac}	Simian immunodeficiency virus from Rhesus macaque
SIV_{rcm}	Simian immunodeficiency virus from Red-capped mangabeys
SIV_{sm}	Simian immunodeficiency virus from Sooty mangabey
sp.	Species

SP1	Signal protein 1
SPRY	SPla and the RYanodine Receptor domain
SSA	Sjogren's syndrome nuclear antigen A
SU	Surface glycoprotein
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
TAR	Trans-activating response element
Tat	Transcriptional activator
TBS	Tris-buffered saline
T-C	Owl monkey TRIMCyp gene
TCR	T-cell receptor
TM	Transmembrane glycoprotein
TRIM	Tripartite motif
TRIMCyp	Fusion protein between tripartite motif protein and cyclophilin A
tRNA	Transfer ribonucleic acid
$tRNA^{Lys}$	Lysyl-transfer ribonucleic acid
TSG101	Tumour susceptibility gene 101
TX	TritonX
U3	Unique 3' region
U5	Unique 5' region
UN	United Nations
UV	Ultra-violet
Vif	Viral infectivity factor
VLP	Virus-like particles
Vpr	Viral protein R
Vps	Vacuolar sorting machinery
Vpu	Viral protein U
Vpx	Viral protein X
V-region	Variable region
VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus glycoprotein
WB	Western blot
WHO	World Health Organisation
wt	Wild type
ZAP	Zinc finger antiviral protein
Zpr1	Zinc finger protein 1

Chapter 1

Introduction

1.1 HIV and AIDS

1.1.1 Discovery

Acquired immunodeficiency syndrome (AIDS) was first recognised in the early 1980s following an increase in the number of people with symptoms of immunologic deficiencies in the United States and Europe. These people were suffering from diseases that had until then, been very rare. They included a variety of unusual cancers (non-Hodgkin's lymphoma and Kaposi's sarcoma), opportunistic infections (*Pneumocystis carinii* pneumonia, *Toxoplasma gondii* encephalitis) and a generalized lymphadenopathy. A common finding in affected individuals was a considerable depletion of the levels of $CD4^+$ T-lymphocytes, a central component of the adaptive immune system, in their peripheral blood.

In June 1981 the Centres for Disease Control (CDC) in Atlanta published a report describing five young homosexual men from California with *Pneumocystis carinii* pneumonia (PCP), which appeared in the *Morbidity and Mortality Weekly Report* (Gottlieb *et al.*, 1981). Reports of rare cases of Kaposi's sarcoma followed (Fannin *et al.*, 1982). All patients were strangely immunosupressed-their immune systems could not fight off even common infections.

The disease still did not have a name at this point and different groups referred to it in different ways. The CDC generally called it by the name of the disease it was causing, for example lymphadenopathy. Because initially, more than 90% of these cases occurred in homosexual or bisexual men the term "gay-related immunodeficiency disease" (GRID) was used. However, by 1982, many new cases had been reported, not only in homosexual men, but also in intravenous drug users, haemophiliacs, blood-transfusion recipients and finally heterosexual adults. The occurrence of the disease in non-homosexuals meant that names such as GRID were redundant and it was suggested that the disease be called AIDS - Acquired Immune Deficiency Syndrome.

In 1983, Luc Montagnier's group at the Institute Pasteur in France reported that they had isolated a new virus, which they suggested might be the cause of AIDS (Barre-Sinoussi *et al.*, 1983). Because this virus was isolated from the lymph node of a man with persistent lymphadenopathy syndrome (LAS) they named it lymphadenopathy-

associated virus or LAV. This virus, a retrovirus, was able to replicate in $CD4^+$ cells and kill them (Montagnier *et al.*, 1984). Similarly, a team led by Robert Gallo from the National Cancer Institute in the United States also reported the characterisation of a human retrovirus, isolated from peripheral blood mononuclear cells from AIDS patients. They called this virus human T lymphotropic virus type III (HTLV-III) (Gallo *et al.*, 1984; Popovic *et al.*, 1984; Sarngadharan *et al.*, 1984; Schupbach *et al.*, 1984). In the hunt for the etiologic agent of AIDS was also Levy's group at the University of San Francisco. They reported the recovery of a retrovirus from AIDS patients, as well as from asymptomatic patients in the various risk groups, which they called AIDS-associated retrovirus (ARV, Levy *et al.*, 1984).

In 1985 a number of more detailed reports were published concerning LAV, ARV and HTLV-III, and it became evident that these viruses were the same and exhibited morphologic and genetic characteristics typical of the *Lentivirus* genus in the *Retroviridae* family of viruses (Marx, 1985; Wain-Hobson *et al.*, 1985). In May 1986, the International Committee on the Taxonomy of Viruses ruled that all those names should be dropped and gave the virus a separate name - HIV (Human Immunodeficiency Virus) (Coffin *et al.*, 1986). It was later modified to HIV-1 with the discovery of a variant of this virus in West Africa, termed HIV-2 (Clavel *et al.*, 1986a, b). Although HIV-2 is also an AIDS-causing agent, it appears to be less pathogenic than HIV-1 and spreads at a slower rate (Whittle *et al.*, 1994).

1.1.2 Occurrence

According to UNAIDS/WHO AIDS epidemic update for December 2006 AIDS has killed more than 25 million people since it was first recognised in 1981 making it one of the most serious infectious disease to have affected humankind in recorded history. The total number of people living with HIV has reached around 40 million and close to 5 million people are infected with the virus every year. Sub-Saharan Africa remains the most affected, with two thirds of all HIV infected people living there, as are 77% of all women with HIV. Growing epidemics are underway in Eastern Europe, Central Asia and East Asia (see Figure 1.1).

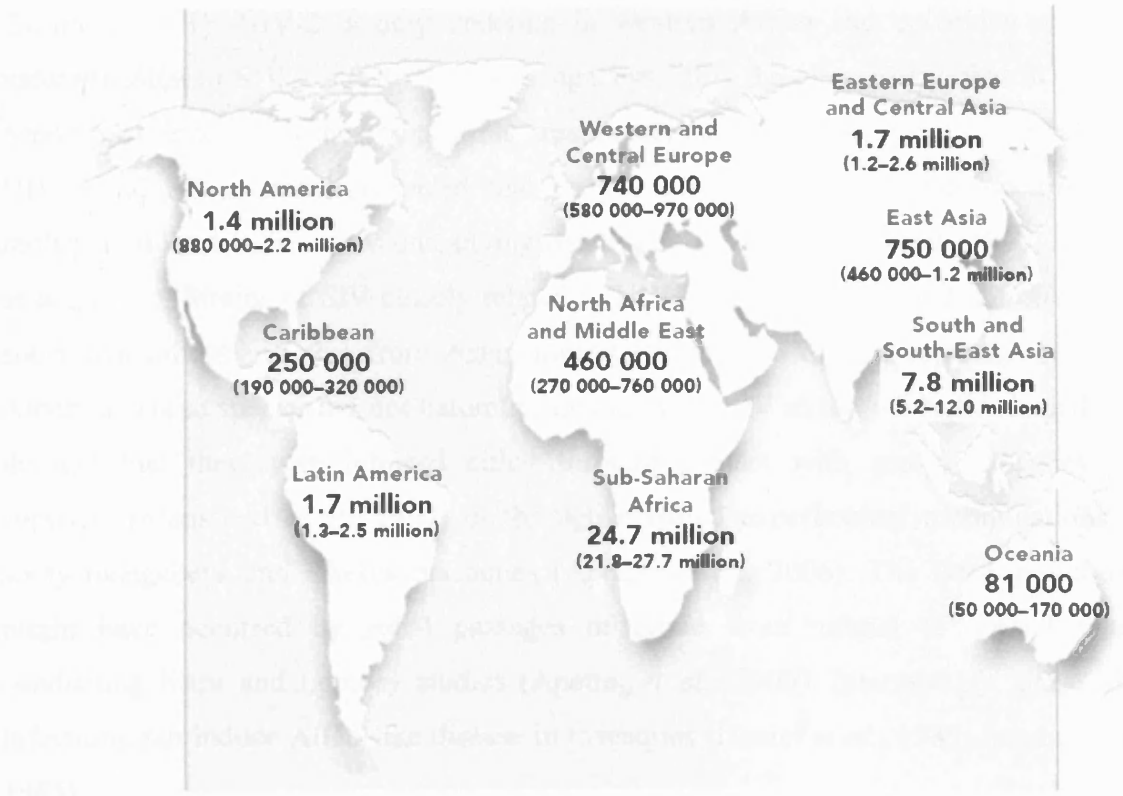


Figure 1.1. Global summary of the AIDS epidemic-December 2006. The numbers represent numbers of infected people. The ranges around the estimates in this figure define the boundaries within which the actual numbers lie, based on the best available information. Source: UNAIDS/WHO AIDS epidemic update, December 2006.

1.1.3 Origin and diversity

Since the discovery of the epidemic causing human viruses HIV-1 and HIV-2, researchers have searched for their origin.

Their closest relatives were found in primates and are collectively referred to as simian immunodeficiency viruses (SIVs). Over twenty primate lentiviruses have been identified in Old World monkeys (family *Cercopithecidae*). SIV infections in their natural host are generally asymptomatic and do not cause immunodeficiency despite high viral loads over long periods of time (Gardner, 2003). The greatest diversity of HIV-1 and HIV-2 viral strains, as well as the highest rates of infection, occurs in sub-Saharan Africa providing compelling evidence that the HIV's have arisen through cross-species transmission from African primates (Peeters and Sharp, 2000) .

Soon after the isolation of HIV-2 from patients in western Africa its primate reservoir was identified as the sooty mangabey (*Cercocebus atys*, Hirsch *et al.*, 1989; Gao *et al.*, 1992). SIV found in these monkeys (SIV_{sm}) was genetically so similar to HIV-2 that some subtypes of HIV-2 are actually closer to SIV_{sm} than they are to each other

(Holmes, 2001). HIV-2 is only endemic in western Africa and coincides with the natural habitat of SIV_{sm} infected sooty mangabeys. It is therefore likely, that SIV_{sm} has been transmitted to humans within this area. Analysis of the phylogenetic trees among HIV-2 and SIV_{sm} strains revealed that cross-species transmission has occurred on multiple (at least eight) occasions, giving rise to groups A-H (Chen *et al.*, 1997; Apetrei *et al.*, 2005). Strains of SIV closely related to HIV-2 have been isolated not only from sooty mangabeys but also from Asian macaque monkeys kept in captivity in North America. These species are not naturally infected with SIV in the wild in Asia and it is thought that they were infected either through contact with sooty mangabeys in captivity (Mansfield *et al.*, 1995) or through invasive experimental manipulations of sooty mangabeys and Rhesus macaques (Apetrei *et al.*, 2006). The latter possibility might have occurred by serial passages of blood from animal to animal while conducting Kuru and Leprosy studies (Apetrei *et al.*, 2006). Interestingly, these SIV infections can induce AIDS-like disease in macaques (Daniel *et al.*, 1985; Letvin *et al.*, 1985).

In contrast to HIV-2, the origin of HIV-1 was less certain. Phylogenetic analysis revealed that the only strains of SIV closely related to HIV-1 are found in chimpanzees (*Pan troglodytes*) (Peeters *et al.*, 1989; Huet *et al.*, 1990). But because until recently, only three infected animals had been found, it was unclear as to whether chimpanzees are really the natural reservoir of these viruses or whether both humans and chimpanzees acquired their lentiviruses independently from a third, as yet unidentified, African monkey. Later careful characterisations of SIVs from numerous primate species as well as from additional SIV_{cpz} strains showed that SIV_{cpz} from the chimpanzee sub-species *Pan troglodytes troglodytes* is the most likely ancestor of HIV-1 (Gao *et al.*, 1999; Keele *et al.*, 2006). Subsequent phylogenetic analysis of the SIV_{cpz} strains from *Pan troglodytes troglodytes* indicated that virus arose from a recombination event among SIV from red-capped mangabeys (SIV_{rcm}) and SIV from greater spot-nosed monkeys (SIV_{gsn}; Bailes *et al.*, 2003). The phylogenetic relationships of primate lentiviruses are shown in Figure 1.2.

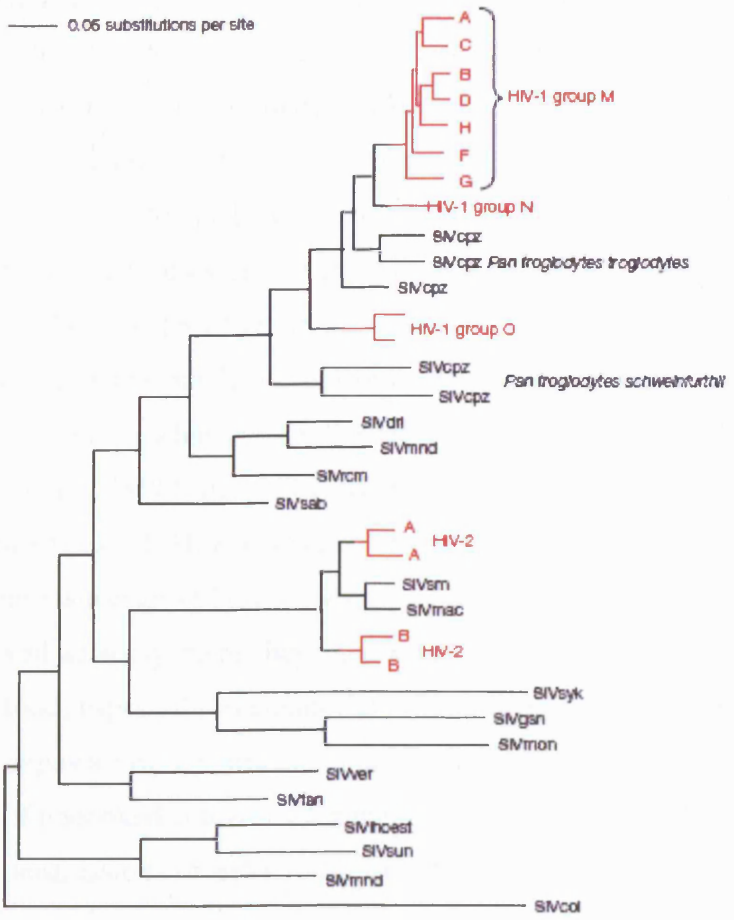


Figure 1.2. Evolutionary history of the primate lentiviruses. Abbreviations for virus primate hosts are as follows: col-colobus; cpz-chimpanzees; drl-drill; gsn-greater spot-nosed monkey; lhoest-L'Hoest monkey; mac-macaque; mnd-mandrill; mon-Campbell's mona monkey; rcm-red caped monkey; sab-Sabaeus monkey; sm-sooty mangabey monkey; sun-sun tailed monkey; syk-Sykes monkey; tan-tantalus monkey; ver-vervet monkey; Reproduced from Rambaut *et al.*, 2004.

Due to its high replication rate, error-prone reverse transcriptase and high frequency of recombination, HIV-1 evolves at an extraordinarily fast rate and has accumulated a large amount of diversity since its introduction into humans. Globally circulating strains of HIV-1 fall into 3 major phylogenetic groups, termed M (main), O (outlier) and N (non M/non O), each resulting from an independent zoonosis into humans. Although isolates from all 3 groups cause AIDS it is the group M which is responsible for the global AIDS pandemic and comprises by far the majority of known HIV-1 isolates. This group had been further subdivided based on the sequence of viral genomes into numerous clades, termed subtypes A-K. Members of HIV-1 group O represent highly divergent strains found mostly in western equatorial Africa. A recent paper reports the discovery of SIVs from wild living gorillas (SIV_{gor}) which are much more closely related to HIV-1 group O than any other known SIV. The phylogenetic relationships

suggest that chimpanzees were the original reservoir of SIVs now found in humans and gorillas giving rise to HIV-1 groups M and N. In case of HIV-1 group O it is unclear at this point if chimpanzees transmitted O-like viruses to gorillas and humans independently or they transmitted it only to gorillas, which then transmitted the virus to humans (Heuverswyn *et al.*, 2006). HIV-1 group N, the latest to be discovered, consists of a very small number of viruses and is thought to be a recombinant between SIV_{cpz} and a virus related to the ancestor of group M (Gao *et al.*, 1999).

The oldest trace of the AIDS pandemic is from a human blood sample taken in 1959 from an adult male living in what is now the Democratic Republic of Congo in west-central Africa (Zhu *et al.*, 1998). In 2000 Korber *et al.* performed a detailed analysis of envelope gene sequences of HIV-1 isolates from more than 150 individuals and estimated the common ancestor of HIV-1 (M-group) in 1931.

Chimpanzees, as well as sooty mangabeys and other primate species, are often hunted and butchered for food, especially in equatorial Africa, and sold at markets as so called "bush meat". This exposure of humans to animal blood and mucosal secretions together with consumption of uncooked contaminated meat provides the simplest explanation for the cross-species transmissions of SIVs to humans (Robinson *et al.*, 1999; Sharp *et al.*, 2001).

1.1.4 Transmission and clinical course of infection

The vast majority of HIV infections are acquired through unprotected sex, both vaginal and anal, when infected sexual secretions of one partner come into contact with the mucous membranes of another. Various mucosal, chemical and physical aspects of the epithelial lining act as a barrier for the initial access of virus to the target cells. These limitations account for the low rate of "successful" sexual transmission of HIV, which is ~1 infection per 1000 exposures through vaginal intercourse (Gray *et al.*, 2001; Varghese *et al.*, 2002; Smith *et al.*, 2005). Direct blood contact is another route of transmission and it accounts for infections in intravenous drug users, hemophiliacs and recipients of blood transfusions and blood products. The third mode of transmission is from mother to child and it can occur before or during birth or through breast milk.

HIV primarily infects cells of the immune system such as $CD4^+$ T-cells, macrophages and dendritic cells. As already mentioned, the disease is associated with a progressive decrease of the $CD4^+$ T-cells and an increase in virus load which are often used to determine a patient's progression of infection. The $CD4^+$ T-cells are destroyed in HIV

infection either directly (through cytopatic effects of HIV virus) or indirectly (through increased rates of apoptosis in infected cells; and through killing of infected $CD4^+$ T-cells by CD8 cytotoxic lymphocytes (Schmitz *et al.*, 1999). Moreover, the ability of immature progenitors to replace $CD4^+$ T-cells is reduced during HIV-1 infection (McCune, 2001; McMichael and Rowland-Jones, 2001).

The first phase of HIV infection, the acute infection, is a stage of rapid viral replication that immediately follows the individual's exposure to HIV, with levels of virus in the peripheral blood approaching several million viruses per ml (Piatak *et al.*, 1993). This is accompanied by a sharp drop in the amount of $CD4^+$ T-cells, which is particularly severe in the gut-associated lymphoid tissue (GALT), where close to 90% of $CD4^+$ T-cells die within the first weeks of infection (Brenchley *et al.*, 2004). The decline in $CD4^+$ T-cells is subsequently associated with the activation of $CD8^+$ T-cells and antibody production. Emergence of latter HIV-specific immune responses and decreased availability of host $CD4^+$ T-cells results in a down-regulation of viraemia and partial recovery of the $CD4^+$ T-cell count. During this period most individuals (80 to 90%) develop an influenza-like illness and sometimes a rash (Kahn and Walker, 1998). Once immune defences lower the number of viral particles in the blood stream, although they do not eradicate the virus, the next stage, so called clinically latent HIV infection begins. This asymptomatic period can vary between 2 weeks and 20 years. Levels of plasma viraemia remain relatively low, which is not due to slower replication of the virus, but to the immune response that kills the infected cells within the body. The $CD4^+$ T-cell count declines slowly throughout this phase. As the $CD4^+$ T-cell count falls below 200 cells/μl (with the normal value being 1200 cells per μl), the risk for opportunistic diseases increases. In the final stages of disease, sharp increases in viraemia and sharp declines in $CD4^+$ T-cell counts occur (Fauci *et al.*, 1996). The clinical course of HIV infection is graphically shown in Figure 1.3. High levels of virus replication, cell destruction and turnover occur throughout the whole course of HIV infection, although it takes years before the $CD4^+$ cell count falls below a critical level for symptomatic immunodeficiency to become manifest. At this stage the immune system collapses, cell-mediated immunity is lost and the body becomes progressively more susceptible to various infections. Eventually, most HIV-infected individuals develop AIDS and die. HIV's case fatality rate is close to 100% (reviewed in detail in Haase *et al.*, 2005).

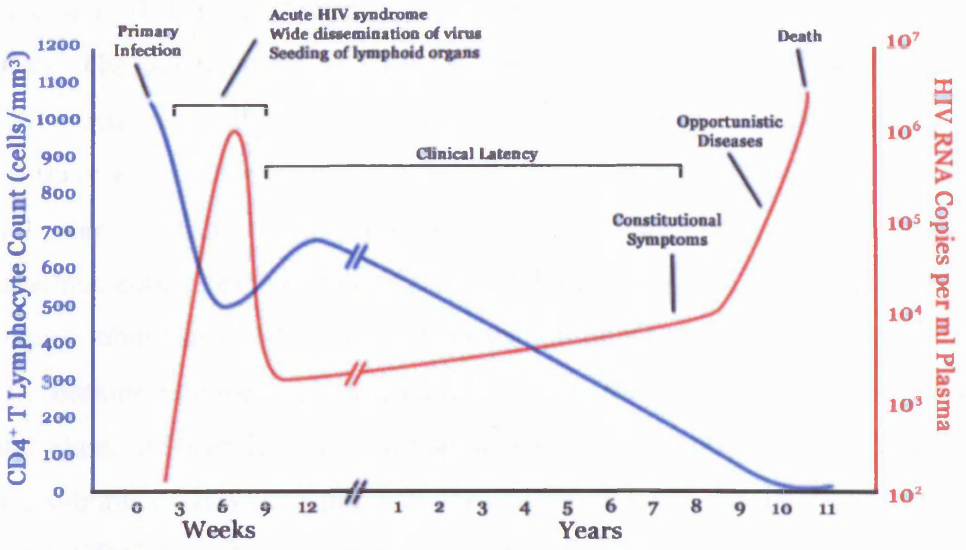


Figure 1.3. The clinical course of HIV infection. A generalized graph of the relationship between HIV copies (viral load) and CD4 counts over the average course of untreated HIV infection; any particular individual's disease course may vary considerably. $CD4^+$ T-cell count (cells per µl)-blue. HIV RNA copies per mL of plasma-red. Fauci *et al.*, 1996.

1.1.5 Vaccines and anti-retroviral therapy

Even after more than 20 years of research, HIV-1 remains a difficult target for a vaccine and there is currently no cure for HIV. For many years attention in vaccine research was focused towards the viral envelope protein and included a variety of approaches such as recombinant proteins, synthetic peptides, recombinant viral or nonviral vectors, recombinant bacterial vectors, recombinant particles, whole-killed or life-attenuated HIV. HIV vaccines have been evaluated in over 70 phase I (dose-escalation safety and toxicity), five phase II (expanded safety and dose optimization) and two phase III (efficacy) trials (Nabel, 2001; Excler, 2005). Unfortunately none of these approaches has been successful. At present, the most advanced candidate in clinical trials is the replication defective adeno-5 vector expressing HIV-1 Gag, which has entered a phase IIB trial in 1500 subjects at risk for HIV-1 infection. Data from this trial will be available in 3-4 years. The proof for the feasibility of HIV vaccine development was provided by the use of live attenuated SIV virus vaccines. They provided complete or near-complete protection from homologous challenge by the same wild-type SIV strains. The mechanism for protection conferred by these vaccines is not fully understood and is currently being extensively studied (Koff *et al.*, 2006).

Even though it is not possible at the present time to prevent the virus from infecting the cells nor clear the body of virus, it is possible, with the use of antiviral drugs, to prevent

the progression of infection towards AIDS and thus to prolong the life of HIV infected individuals. The development of antiviral drugs required the identification of targets within the virus whose interference with, would render the virus non-infectious. The enzymes reverse transcriptase and protease became the first targets. Currently, 20 anti-retroviral agents from four classes are available (Table 1.1). Apart from nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs), a novel group of fusion inhibitors became commercially available in 2003 (Kalkut, 2005). The single drug in this latter class, enfuvirtide is a 36-amino-acid synthetic peptide that binds to the gp41 envelope subunit of HIV and prevents the conformational change necessary for HIV fusion with $CD4^+$ cells. It is the only drug within the anti-retroviral agents delivered by injections (Manfredi and Sabbatani 2006, more about fusion in chapter 1.2.3.1). This pharmacological class is probably going to expand after the completion of several phase II-III trial, with inhibitors of HIV co-receptors. A current focus is the development of integrase inhibitors. Several classes of HIV integrase inhibitors have been reported (reviewed in Makhija, 2006). The most promising, called raltegravir, is an HIV-1 integrase strand transfer inhibitor that has been shown to be active against multidrug-resistant HIV-1. It is now being evaluated in phase II dose-ranging study (Grinsztejn *et al.*, 2007).

At the present time antiviral treatment for HIV consists of, so called, highly active anti-retroviral therapy (HAART). Because usage of only one specific drug at a time proved to be ineffective in the long term due to the fast mutation rate of HIV, current HAART is a combinations (or "cocktail") of at least three drugs belonging to at least two classes of anti-retrovirals. Typically, these are two nucleoside analogue reverse transcriptase inhibitors plus either a protease inhibitor or non-nucleoside reverse transcriptase inhibitor. Although HAART stabilises a patient's symptoms and viraemia, high levels of HIV-1, often HAART resistant, return if treatment is stopped (Martinez-Picado *et al.*, 2000; Dybul *et al.*, 2002). Despite this, the use of this therapy has led to a large reduction in HIV-associated illness and death in the developed world and improved the health and quality of life of many HIV-infected individuals.

Generic name	Manufacturer	Brand name
Nucleoside nucleotide reverse transcriptase inhibitors (NRTIs)		
1987		
Zidovudine (AZT)	GlaxoSmithKline	Retrovir
Didanosine (ddI)	Bristol-Myers Squibb	Videx
Zalcitabine (ddC)	Roche Pharmaceuticals	Hivid
Stavudine (d4T)	Bristol-Myers Squibb	Zerit
Lamivudine (3TC)	GlaxoSmithKline	Epivir
Abacavir (ABC)	GlaxoSmithKline	Ziagen
Tenofovir (TDF)	Gilead Sciences	Viread
Emtricitabine (FTC)	Gilead Sciences	Emtriva
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)		
1996		
Nevirapine (NVP)	Boehringer Ingelheim	Viramune
Delavirdine (DLV)	Pfizer	Rescriptor
Efavirenz (EFZ)	Boehringer Ingelheim	Sustiva
Protease inhibitors (PIs)		
1995		
Saquinavir (SQV) hard and soft gel	Roche Pharmaceuticals	Invirase Fortavase
Ritonavir (RTV)	Abbott Laboratories	Norvir
Indinavir (IDV)	Merck	Crixivan
Nelfinavir (NFV)	Pfizer	Viracept
Amprenavir (APV)	GlaxoSmithKline	Agenerase
Atazanavir (ATV)	Bristol-Myers Squibb	Reyataz
Fosamprenavir (fAPV)	GlaxoSmithKline	Lexiva
Lopinavir (LPV)	Abbott Laboratories	Kaletra
Fusion inhibitors		
2003		
Enfuvirtide (T-20)	Roche Pharmaceuticals	Fuzeon

Table 1.1. Anti-retroviral agents approved by the U.S. Food and Drug Administration (May 2006). The name of manufacturer, brand name and year of development of first representative of each anti-retroviral class is included. Adapted from Makhija, 2006; Manfredi and Sabbatani, 2006.

1.2 The Molecular Biology of HIV

1.2.1 Taxonomy

The retrovirus family, the *Retroviridae*, is a large and diverse family of enveloped RNA viruses found in all vertebrates. Retroviruses are associated with a wide variety of diseases in mammals including malignancies, immune deficiency and central nervous system defects.

Retroviruses are broadly divided into 2 categories: simple and complex, depending on the organization of their genomes. The simple viruses encode only the Gag, Pro, Pol, and Env gene products; the complex viruses encode these same gene products but also an array of additional proteins derived from multiply spliced messages with a range of functions. Retroviruses are divided into 7 genera: alpharetroviruses, betaretroviruses, and gammaretroviruses are considered "simple" retroviruses, whereas the deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaviruses are considered "complex." Some of the best studied retroviruses are Murine leukaemia viruses (MLVs) belonging to the gammaretrovirus genus and human immunodeficiency viruses 1 and 2 (HIV-1, HIV-2) from the Lentivirus genus. Representative members of each genus are listed in Table 1.2.

Subfamily	Genus	Examples
Orthoretrovirinae	Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)
	Betaretrovirus	Mouse mammary tumour virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retrovirus (JSRV)
	Gammaretrovirus	Murine leukaemia viruses (MoMLV) Feline leukaemia virus (FeLV) Gibbon ape leukaemia virus (GaLV) Reticuloendotheliosis virus (RevT)
	Deltaretrovirus	Human T-lymphotropic virus (HTLV)-1,-2 Bovine leukaemia virus (BLV) Simian T-lymphotropic virus (STLV)-1,-2,-3
	Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1
	Lentivirus	Human immunodeficiency virus 1,2 (HIV-1, HIV-2) Simian immunodeficiency virus (SIV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus
Spumaretrovirinae	Spumavirus	Human foamy virus (HFV)

Table 1.2. Taxonomy and representative members of family *Retroviridae*. Adapted from Goff, 2001a.

1.2.2 The HIV virion and genome structure

The mature HIV particle is an enveloped spherical structure of about 120 nm in diameter with an enclosed conical-shape capsid composed of around 2,000 molecules of the viral capsid protein. The viral genome within the capsid is composed of two copies of linear, positive, single-stranded RNA with a length of approximately 9-kilobases. The virions are therefore functionally diploid (Figure 1.4).

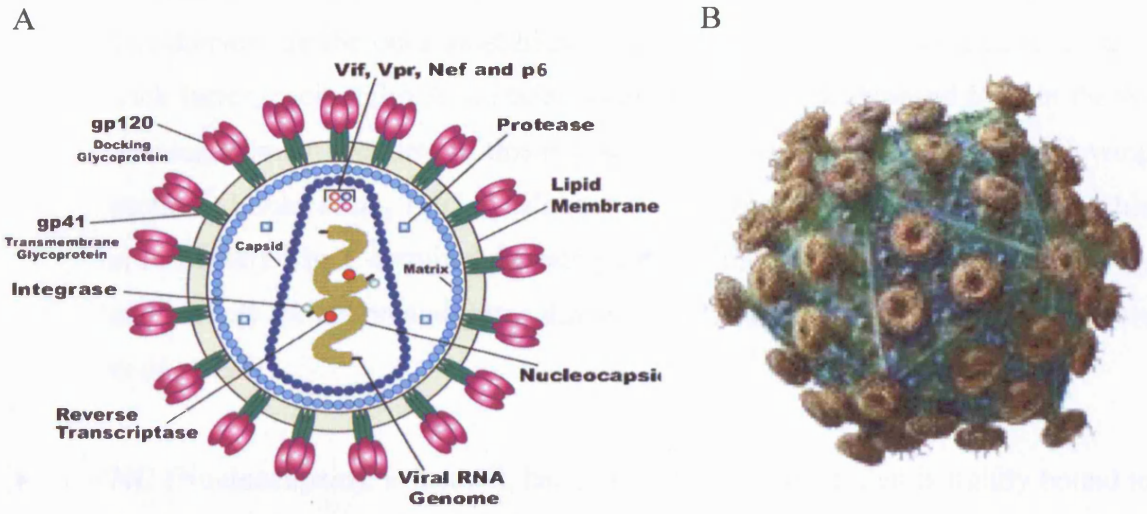


Figure 1.4. Schematic drawing of the HIV virion. A- Stylised rendering of a cross section of the HIV; B- Schematic drawing of HIV virion surface. Taken from http://en.wikipedia.org/wiki/HIV and http://www.rkm.com.au/imagelibrary

The HIV-1 RNA molecule contains nine open reading frames which encode fifteen proteins (Figure 1.5; reviewed in Frankel and Young, 1998; Coffin *et al.*, 1997; Goff, 2001a; Anderson and Hope, 2003):

1) Gag (group-specific antigen) polyprotein (p55) is a 55-kilodalton precursor protein which is cleaved into structural virus proteins: matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6.

- **MA (Matrix)**: The viral structural protein derived from the amino-terminal domain of the Gag polyprotein. MA is associated with the inner surface of the virus envelope surrounding the capsid. Matrix protein has been implicated in the nuclear import of HIV-1 preintegration complexes although it is not essential (Bukrinsky *et al.*, 1993b; von Schwedler *et al.*, 1994; Fouchier *et al.*, 1997; Reil *et al.*, 1998). It undergoes myristylation at amino-acid position 2 (always Gly)

that targets the Gag polyprotein to membranes during viral assembly (Gottlinger *et al.*, 1989; Bryant and Ratner 1990; Pal *et al.*, 1990).

- **CA (Capsid)**: This principal structural protein forms the cone-shaped core of the virus particle that encloses the viral RNA, enzymes, and accessory proteins necessary for infecting the host cell. The HIV-1 CA contains distinct N- and C-terminal domains separated by a short flexible linker (Gitti *et al.*, 1996). The N-terminal domain forms the outer surface of the CA core and exposed residues in this domain are the ones most likely to interact with cellular host factors. One such factor, cyclophilin A, associates with Pro90 on the exposed loop in the N-terminal domain (features of this interaction are expanded upon in the following sections, Luban *et al.*, 1993; Thali *et al.*, 1994; Braaten *et al.*, 1996b, c; Gamble *et al.*, 1996). The C-terminal domain plays an important role in capsid assembly and particle formation and forms dimers in solution and within crystals (Gamble *et al.*, 1997).

- **NC (Nucleocapsid)**: is a small, basic, zinc-finger protein that is tightly bound to the genomic RNA in the core. It binds specifically to the packaging signal and delivers the full-length viral RNAs into the assembling virion.

- **p6**: Two functions have been proposed for this proline-rich, 6-kDa protein: incorporation of the HIV-1 accessory protein Vpr into virus and a role in virus particle production. It contains the late domain (PTAP) that is necessary for the final steps of virion budding (Huang *et al.*, 1995).

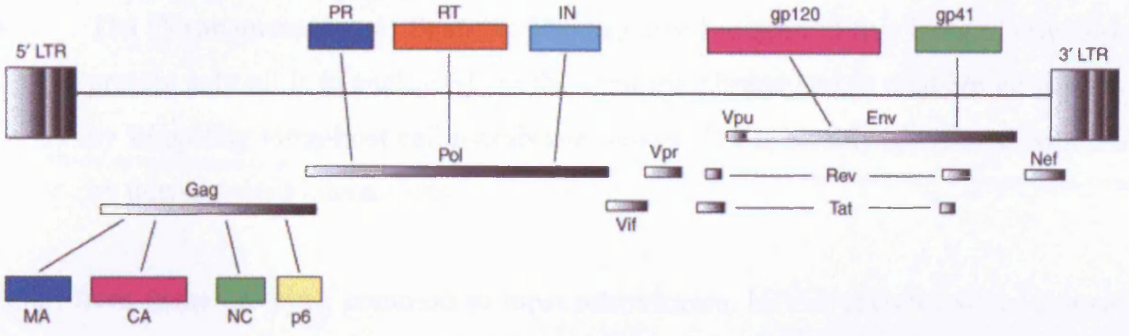


Figure 1.5. Organisation of the HIV-1 genome. The location of the long terminal repeats (LTRs) and the genes encoded by HIV-1 are indicated. Fifteen viral proteins encoded by HIV-1 are shown in rectangles. The sizes of the genes and encoded proteins are not to scale. Freed, 2004.

2) Pol Polyprotein; a precursor protein which is processed into 3 viral enzymes: protease (PR, p10), reverse transcriptase (RT, p66/51), and integrase (IN, p32).

- **PR (Protease)**: Cleaves the Gag and Gag-Pol polyproteins to produce viral proteins in their mature forms. Retroviral proteases function as homodimers.

- **RT (Reverse Transcriptase)**: An enzyme that catalyses the synthesis of the viral RNA into double-stranded DNA. Its carboxy-terminal domain contains an RNase H activity that cleaves the RNA portion of the RNA-DNA hybrid generated during the reverse transcription reaction (Tanese and Goff, 1988). HIV-1 RTs are heterodimers.

- **IN (Integrase)**: DNA recombinase responsible for insertion of the linear double-stranded DNA copy of the retroviral genome into the host chromosome thus forming a provirus. HIV-1 integrase forms tetramers (Cherepanov *et al.*, 2003).

3) Env Polyprotein; is a viral glycoprotein produced as a precursor (gp160) which is cleaved into surface glycoprotein (SU, gp120) and transmembrane glycoprotein (TM, gp41).

- **SU (Surface) Protein**: trimers of this protein are located on the surface of the virus and bind to specific cell-surface receptors (CD4 in the case of HIV) and co-receptors. Binding of CD4 to SU cause structural changes to the virus that facilitate co-receptor binding and subsequent viral entry.

- **TM (Transmembrane) Protein**: The primary function of this integral envelope protein subunit is to anchor SU in the viral membrane and to mediate virus entry by triggering virus-host cell membrane fusion. TM is closely associated with SU by non-covalent interactions.

Apart from these proteins, common to most retroviruses, HIV-1 encodes six additional proteins, often called accessory proteins, three of which (Vif, Vpr, and Nef) are found in the viral particle. An overview of these proteins is in table 1.3 and a more detailed description of their function is provided in chapter 1.2.3.

NAME	SIZE	FUNCTION
Tat (Transcriptional activator)	p16/ p14	Essential for virus replication. Activates viral transcription by binding to the TAR RNA element of newly transcribed provirus and recruits several host factors that enhance RNA polymerase II elongation on the viral DNA template.
Rev (Regulator of viral gene expression)	p19	Phosphoprotein that binds to the RRE site (a site located in the *env* coding region) and promotes nuclear export of unspliced and incompletely spliced viral RNAs to the cytoplasm.
Vif (Viral infectivity factor)	p23	Allows HIV-1 to evade an innate immune defence in the host cell mediated by members of the APOBEC family of proteins. Vif targets APOBEC3G for proteasomal degradation thus preventing it from entering the virion during budding from a host cell.
Vpr (Viral protein **R**)	p14	Basic protein, found in virions, causes infected cells to arrest in the G_2 phase of the cell cycle and may target retroviral preintegration complexes to the nucleus.
Vpu (Viral protein **U**)*	p16	An integral membrane protein that causes degradation of newly synthesised CD4 in the endoplasmic reticulum and enhancement of virion release from the plasma membrane. The latter is possibly due to counteracting a dominant host cell restriction of HIV-1 particle production (Varthakavi *et al.*, 2003; Neil *et al.*, 2006). Vpu is unique to HIV-1 and SIV_{cpz}.
Nef (Negative factor)	p27	A myristylated protein that reduces the levels of cellular CD4 and MHC I molecules, upregulates FasL expression, enhances virion infectivity and alters the state of cellular activation. HIV-1 Nef is lacking the ability to downregulate TCR-CD3 and thus to inhibit cell death. This ability is common to Nef's from other primate lentiviruses, including HIV-2, resulting in a decreased pathogenicity of the virus (Schindler *et al.*, 2006). HIV pathogenesis is slowed significantly in the absence of Nef. It is also hypothesised that Nef prevents virus from being restricted by a proteasome-dependent cellular mechanism (Qi and Aiken, 2007).

Table 1.3 Accessory proteins of HIV-1. *Comparing to HIV-1, HIV-2 does not encode Vpu but contains an additional gene called Vpx (Viral Protein X). Two functions of Vpr in HIV-1 are split between Vpr and Vpx in HIV-2, with the HIV-2 Vpr protein inducing cell cycle arrest and the Vpx protein required for nuclear import.

HIV RNA contains a number of special RNA elements which are required for viral replication. These key sequences are mostly clustered at the termini of the RNA (Figure 1.6). Starting from the 5' end immediately after the cap is the TAR hairpin. This is the Tat-binding site followed by a short sequence, called R (for repeated) region. It is present twice in the RNA, at both RNA termini. Downstream of R sequence lies U5 (unique 5') region, which includes one of the *att* sites needed for proviral integration. Next follows the primer binding site (pbs) at which a tRNA is hybridized to the viral genome and serves as a primer for the initiation of minus-strand DNA synthesis by reverse transcription. The region downstream of the pbs contains the major signal for the encapsidation of viral RNA into the virion. It is called the Psi (ψ) element or packaging sequence. This region also contains a "kissing loop" hairpin which is a dimerisation site that facilitates incorporation of 2 genomic RNAs into the virion and a major splice donor site which is needed for the formation of subgenomic RNAs. The

following RNA sequences are coding regions for the viral proteins and include the slippery sequence and hairpin that promotes the ribosomal frameshift needed for translation of the HIV-1 Gag-Pol polyprotein. An approximately 200 nucleotide RNA element called the RRE (Rev Response Element) is encoded within the *env* region of HIV-1. The RRE contains a high affinity binding site for Rev and is necessary for Rev function. Downstream of the coding genes lie most of the splice acceptor sites and a polypurine tract (ppt), a short site of at least nine A and G residues that is important for the initiation of the plus-strand DNA synthesis (lentiviruses also contains a central copy of the ppt region (cppt) in the centre of the genome). It is followed by a unique 3' sequence (U3) which contains a number of important *cis*-acting elements for viral gene expression and another of the *att* sites required for DNA integration. Almost at the end of the 3' end is the 3' copy of the R region followed by a poly(A) tail.

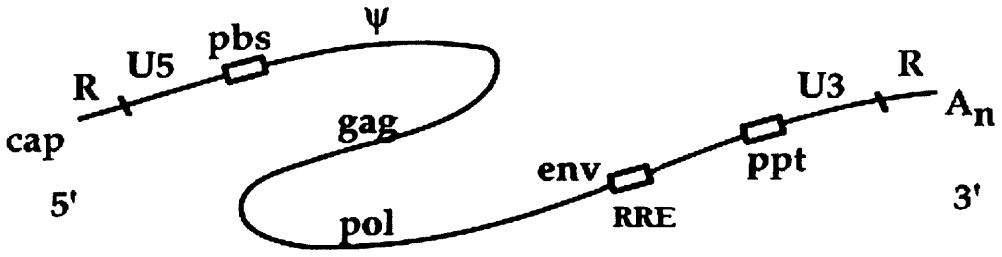


Figure 1.6. The structural elements of the retroviral RNA genome. The single-stranded RNA genome is depicted as a *curved line*. From 5′ to 3′ along the RNA, the features include a 5′ cap structure; *R*, a sequence block repeated at both 5′ and 3′ ends; *U5*, a unique 5′ sequence; *pbs*, the primer binding site and site of initiation of minus-strand DNA synthesis; *Y*, the major recognition site for the packaging of the viral RNA into the virion particle; the *gag*, *pol*, and *env* genes; RRE, the Rev response element; *ppt*, the polypurine tract and site of initiation of the plus-strand DNA synthesis; *U3*, a unique 3′ sequence; the second copy of the R sequence; and finally, a 3′ poly(A) sequence. See text for details. Goff, 2001a.

1.2.3 The HIV life cycle

As intracellular parasites, retroviruses rely heavily on the use of numerous cellular machineries for completion of their replication cycle. This section describes the life cycle of HIV and considers positive cellular factors necessary for viral replication. Examples of dominant negative cellular factors that inhibit retroviral infection are described in chapter 1.3.

Retroviruses replicate through a unique life cycle, which differentiates them sharply from other viruses (Figure 1.7). The life cycle of a retrovirus starts with the binding of particles to the host cell membrane. Fusion of the viral envelope with the cellular

plasma membrane, via specific receptor-envelope interactions, permits the viral core to enter the infected cell. The viral reverse transcriptase is then used to transcribe genomic RNA into a double-stranded DNA molecule. After nuclear translocation, viral DNA is inserted into the host-cell chromosome by viral integrase and is then called a provirus. This integration is required for the synthesis of new viral RNA molecules by the host cell RNA polymerase. At this point, the early phase of the viral life cycle ends and the late phase begins, where transcription and translation of viral DNA are performed by the cellular machinery. New viral RNA strands serve either as viral RNA genomes or as mRNA that is translated into viral proteins. After assembly of RNA and viral proteins, particles bud from the plasma membrane and are further matured by protease cleavage of Gag polyprotein. Retroviruses are examples of enveloped viruses, in which the protein shell is enclosed by an outer lipid bilayer membrane (reviewed in Coffin *et al.*, 1997, Goff, 2001a).

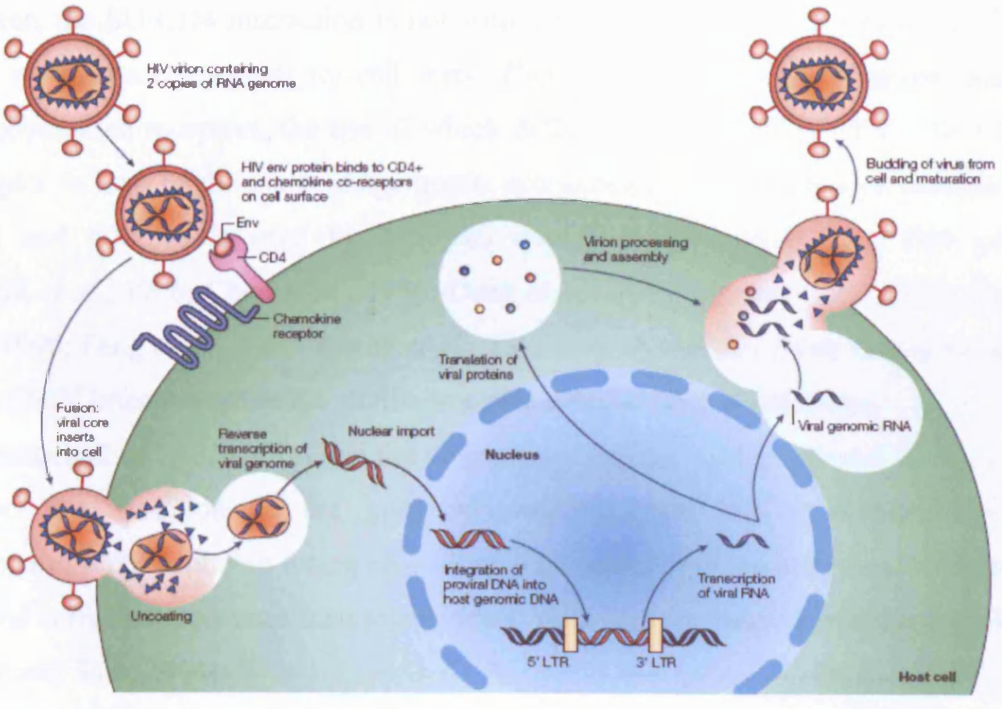


Figure 1.7. A schematic view of the retrovirus life cycle. Indicated are the major steps of HIV-1 replication. See text for details. Rambaut *et al.*, 2004.

1.2.3.1 Binding and virus entry

HIV begins its replication cycle by the adsorption of viral particles to the surface of their target cell. This early attachment of the virions to the cell surface has been attributed to variety of cell-surface molecules, including heparan sulphate (Mondor *et*

al., 1998) and nucleolin (Nisole *et al.*, 1999). HIVs and SIVs are also known to bind dendritic cells through interaction of their envelope glycoproteins with DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and DC-SIGNR (DC-SIGN related) molecules on the surface of dendritic cells (Pohlmann *et al.*, 2001). These attachment factors do not promote viral entry and lead to productive infection, but instead, serve to concentrate the virus on the target cell surface prior to its interaction with a specific viral receptor or to transport the virus to new compartments within the host.

Following binding, retroviral particles, through interactions of viral envelope glycoproteins, use specific host receptors to enter the target cell. The main cell surface molecule to which the HIV surface protein binds is CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986). This cell-surface glycoprotein is present on cells of the immune system: T lymphocytes, monocytes, macrophages and antigen presenting dendritic cells which makes these cells the prime target for infection. However, the SU-CD4 interaction is not sufficient for HIV entry. In addition to CD4, HIV-1 requires a co-receptor for cell entry. There are 2 types of co-receptors, both of them chemokine receptors, the use of which defines two 'groups' of HIV. The CCR5 coreceptor is used by the macrophage-tropic, non-syncytium-inducing viral isolates (R5 group) and CXCR4 is used by T-tropic, syncytium-inducing isolates (X4 group, Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996; Zhang *et al.*, 1996). R5 strains dominate during the early years of HIV infection while X4 strains sometimes occur later in infection.

The interaction of gp120, CD4 and the co-receptor triggers conformational changes that lead to the transition of the gp41 envelope subunit into a fusion-competent conformation. Hydrophobic fusion sequences are inserted into the target cell membrane, viral and cellular membranes fuse together and viral core is released into the cytoplasm (Nisole and Saib, 2004).

1.2.3.2 Uncoating

Once the viral core, containing the viral RNA genome and assorted proteins, is released into the cytoplasm, it undergoes at least partial disassembly (a process usually referred to as uncoating) before reverse transcription can be successfully completed. This point in the retroviral life cycle is poorly understood. Capsid uncoating leads to the formation of sub-viral particles called reverse transcription complexes (RTCs) and pre-integration

complexes (PICs). Typically RTCs are complexes where reverse transcription takes place and PICs are integration-competent complexes with completed reverse transcription product. Both are large nucleoprotein structures. Biochemical analysis of the RTCs/PICs is technically difficult and has failed to provide a clear answer about the RTC/PIC's protein composition. The problem is that the size, components and density of RTC are very sensitive to detergents and thus depend on the conditions used for their isolation. Another problem is that the majority of the viral particles entering the cell will not cause productive infection and thus their purified complexes may not necessarily represent the composition of infectious RTCs/PICs.

Most studies have shown that HIV-1 PICs contain the *pol* gene products (protease, reverse-transcriptase, integrase) and Vpr. The presence of the Gag proteins within these complexes is less clear. Earlier studies indicated that nucleocapsid and matrix proteins were present in purified PICs (Bukrinsky *et al.*, 1993a, b; Miller *et al.*, 1997) whereas a more recent report contradicts this (Fassati and Goff, 2001). However, most studies agree that PICs purified from HIV-1 infected cells are not associated with CA protein while murine leukaemia virus PICs appear to contain CA. MLV cores persist longer than those of HIV since, apart from CA, also MLV NC and MA are detected in the vicinity of the nuclear membrane (Risco *et al.*, 1995). HIV-1 RTCs/PICs seem to recruit cellular proteins, for instance cellular Ku70, Ku90, HMG A1, LEDGF and BAF (more about these proteins in chapter 1.2.3.6; reviewed in Dvorin and Malim, 2003).

Little is known about how viral core disassembles and which of the viral or cellular proteins influence this event. Only one host protein, cyclophilin A, has been convincingly shown to functionally interact with the HIV-1 CA. CypA is an abundantly expressed cell protein that catalyses the *cis/trans* isomerisation of peptidyl-prolyl bonds and which was shown to be incorporated into HIV-1 virions through its interaction with the G89-P90 residues in a protruding CA loop (Luban *et al.*, 1993; Franke *et al.*, 1994; Thali *et al.*, 1994; Gamble *et al.*, 1996). It is estimated that roughly one molecule of CypA is packaged per 10 Gags (Cantin *et al.*, 2005). The failure of HIV-1 CA to bind CypA leads to a defect in the early phase of HIV-1 infection, before reverse transcription (Franke *et al.*, 1994; Thali *et al.*, 1994; Braaten *et al.*, 1996a, b, c; Dorfman and Gottlinger, 1996; Franke and Luban, 1996; Braaten and Luban, 2001; Sokolskaja *et al.*, 2004). The precise functional role of CypA in infection remains to be determined. Several groups have hypothesised that CypA functions as an uncoating factor and has a role in the disassembly of the CA core (reviewed in Luban, 1996). However, later it was suggested that CypA prevents an antiviral factor from accessing

the viral capsid (Towers *et al.*, 2003; for further details see chapter 1.3.4). The role of CypA in HIV-1 replication is the subject of Result Chapter 2.

1.2.3.3 Reverse transcription

In vitro assays have demonstrated that addition of the four dNTPs and a divalent cation to HIV virion is all that is required for the reverse transcriptase to start DNA synthesis (Zhang *et al.*, 1998; Zhang *et al.*, 2000). Although reverse transcription, can initiate within the intact core it seems that for the progression of reverse transcription the uncoating of the viral core is essential (Zhang *et al.*, 2000). HIV-1 reverse transcription occurs within RTCs during its transport through the cytoplasm of infected cell to the nucleus.

As a schematic representation of this process shows in Figure 1.8., HIV reverse transcription is initiated by the annealing of a $tRNA^{Lys}$ primer to a pbs site of the genomic RNA. It then proceeds through a complex series of steps and ends with formation of a linear, double stranded DNA molecule with a U3, R and U5 repetitions, called long terminal repeats (LTRs), at each end of a new DNA molecule. At this point the newly formed cDNA is ready for integration.

Very few host factors were shown to be important for the process of reverse transcription. Some studies have revealed that an intact actin cytoskeleton is important for efficient reverse transcription (Bukrinskaya *et al.*, 1998). Kewalramani and colleagues recently reported that expression of a C-terminally truncated form of murine CPSF6, a component of spliceosomes and 3'pre-mRNA processing machinery, restricted infection by X4- and R5-tropic HIV-1, HIV-2, and SIV but not by MLV. The block occurs at the later stages of reverse transcription when the completion of second strand synthesis is decreased while the early steps of reverse transcription are unaffected. The wild-type form of CPSF6 did not interfere with HIV-1 infection (Ambrose *et al.*, *in press*).

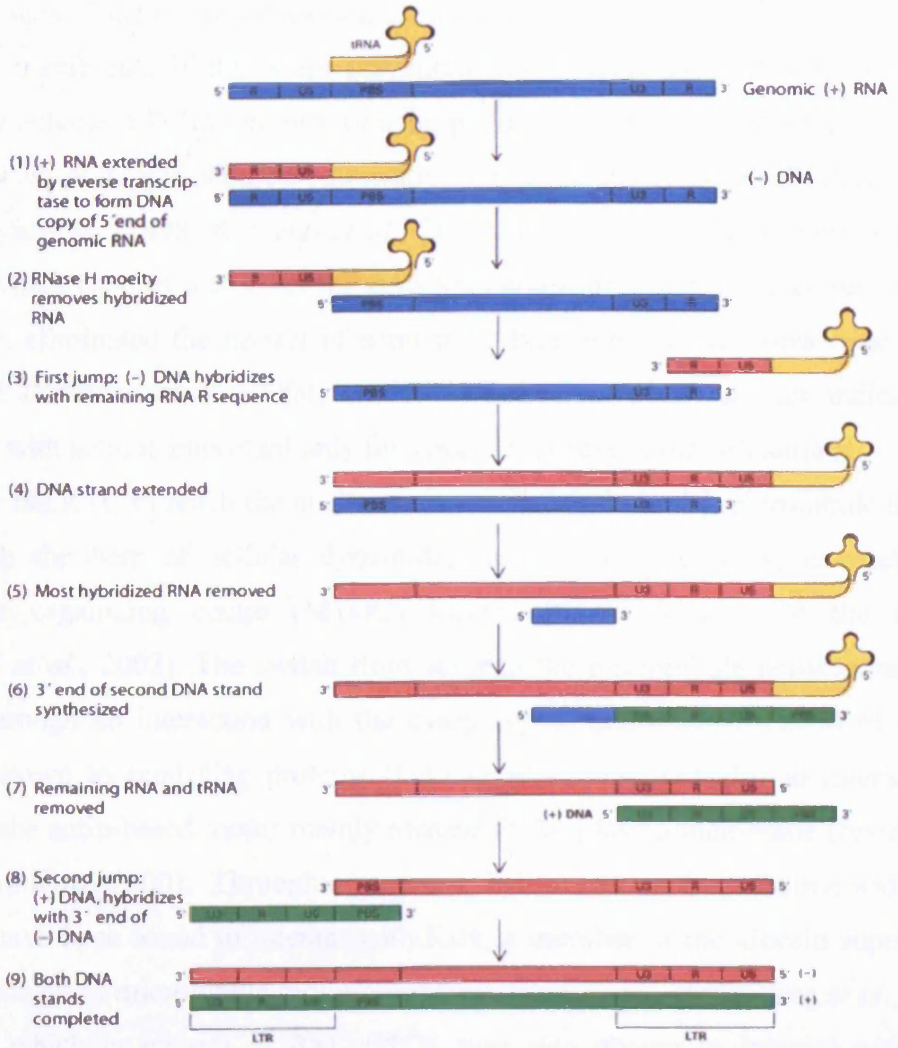


Figure 1.8. The reverse transcription of the retroviral genome. *Yellow and blue* colours represent RNA; *red and green* colours represent DNA. Adapted from www.jyi.org/articleimages.

1.2.3.4 Trafficking of incoming virus through the cytoplasm

To reach the pores in the nuclear membrane, the RTCs have to travel through the cytoplasm to the nucleus. Because of the high viscosity of the cytoplasm, due to the presence of organelles, the cytoskeleton and the high protein concentration, significant movement of these large particles by simple diffusion is unlikely. To overcome this obstacle, HIV-1 utilises the cellular cytoskeleton (reviewed in Dvorin and Malim, 2003).

After the fusion of the viral and cellular membranes, HIV RTCs have been shown to initially associate with the actin filaments which may involve a direct interaction between matrix and/or nucleocapsid protein and the actin network (Bukrinskaya *et al.*, 1998; Liu *et al.*, 1999; Wilk *et al.*, 1999; Ibarrondo *et al.*, 2001). Although actin is

present throughout the entire cytoplasm, its concentration is highest directly underneath the plasma membrane. If RTCs are prevented from interacting with actin filaments, either by cytochalasin D, an inhibitor of actin polymerization or by blocking the Arp2/3 complex which promotes actin polymerisation, viral infectivity is significantly reduced (Bukrinskaya *et al.*, 1998; Komano *et al.*, 2004). Pseudotyping HIV-1 with a VSV-G envelope, which enables it to enter the cells via endosomes, instead of by fusion at the cell surface, eliminated the impact of actin microfilaments (Bukrinskaya *et al.*, 1998) and Arp2/3 (Komano *et al.*, 2004) on HIV-1 infection. These studies indicate that association with actin is important only for viruses that fuse at the cell surface.

In order for the RTC to reach the nucleus it is translocated via the microtubule network where, with the help of cellular dynein-dependent motor complex, it reaches the microtubule organizing centre (MTOC) located in the vicinity of the nucleus (McDonald *et al.*, 2002). The switch from actin to the microtubule network might be provided through an interaction with the cytoplasmic light chain 8 (LC8) of dynein. LC8 was shown to bind Gag proteins (Petit *et al.*, 2003) and also to interact with myosin V, the actin-based motor mainly located at the plasma membrane (reviewed in Harrison and King, 2000). Through a yeast-two hybrid screen, Gags from a wide range of viruses have been found to interact with Kif4, a member of the kinesin superfamily and a component of microtubule motor complexes (Kim *et al.*, 1998; Tang *et al.*, 1999). HIV-1 IN, which is present in RTCs/PICs, was also shown to interact with yeast microtubule-associated proteins (de Soultrait *et al.*, 2002). HIV-1 is not the only virus that utilises the microtubule network for intracellular transport—a similar mechanism has been demonstrated for other viruses such as herpes simplex virus-1 (Sodeik *et al.*, 1997) and adenovirus (Suomalainen *et al.*, 1999; Kelkar *et al.*, 2004).

Recently, a published report presented data demonstrating that cyclophilin A associates in vitro and in vivo with dynamitin, a component of the dynein/dynactin motor protein complex (Galigniana *et al.*, 2004). These results suggest that CypA may act as a connector between the HIV-1 CA core and the microtubule network. However, the experiments demonstrating this link have not been reported.

1.2.3.5 Nuclear import

Reverse-transcribed DNA associated with viral proteins in the form of PICs must enter the nucleus in order to proceed with the infectious cycle. PICs from most retroviruses (like MLV) are unable to enter intact nuclei and must wait for the breakdown of the

nuclear envelope occurring during mitosis (Roe *et al.*, 1993). In contrast, lentiviruses are able to actively cross nuclear membranes allowing them to productively infect non-dividing cells such as macrophages (Weinberg *et al.*, 1991; Lewis and Emerman, 1994). HIV-1 PICs are too large to diffuse through nuclear pore complexes (NPCs), a large supramolecular protein structures that span the nuclear membrane. Instead, HIV-1 PICs use an active, energy-dependent mechanism for nuclear import (Bukrinsky *et al.*, 1992). Some of the components of HIV-1 PICs contain a nuclear localization signal/s (NLS) that engages the cellular transport proteins, which then direct the PIC through the nuclear pore. The NLS in the PIC most likely interacts with the nuclear receptor importin β, either directly or through the adapter importin α or another adaptors such as snurportin, RIP (Rev interacting protein) or importin 7 (Fried and Kutay, 2003). Although importin 7 has been proposed to play a role in the nuclear import of HIV-1 PICs in primary macrophages (Fassati *et al.*, 2003) a subsequent study failed to confirm this finding (Zielske and Stevenson, 2005).

Initially it was thought that the most likely protein mediators of the uptake of HIV-1 PIC into the nucleus were the HIV-1 MA, IN and Vpr proteins associated with the PIC. All of them were shown to contain one or atypical or non-typical nuclear localisation signals and to interact with importins (reviewed in Goff, 2001b; Bukrinsky, 2004; Nisole and Saib, 2004; Anderson and Hope, 2005; Fassati, 2006). Another factor proposed to regulate HIV nuclear import is the unusual structure of the viral cDNA intermediate called the "central DNA flap" structure (Zennou *et al.*, 2000). It is a triple stranded DNA structure which is produced because of additional initiation of the plus-strand DNA synthesis at a central polypurine tract of the HIV genome (within *pol* sequence). It may provide the optimal conformation to the PIC necessary for interaction with importins and/or translocation through the nuclear pore (Zennou *et al.*, 2000; Van Maele *et al.*, 2003; Ao *et al.*, 2004). However, the importance of each above mentioned viral protein in nuclear translocation remains controversial since subsequent studies have shown that HIV lacking one or more of these NLS elements still retains a significant ability to infect non-dividing cells (Fouchier *et al.*, 1997; Reil *et al.*, 1998; Petit *et al.*, 2000; Limon *et al.*, 2002). Moreover, even when all of these viral NLS elements were impaired at once (either by mutation, deletion or replacement with MLV analogue) a resulting chimeric HIV-1 was still able to infect non-dividing cells (Yamashita and Emerman, 2005). Thus, elucidating the mechanism of HIV-1 nuclear import is clearly a challenging area of research and it still remains to be fully characterised.

1.2.3.6 Integration

Once within the nucleus, integration of reverse transcribed viral cDNA is carried out by the integrase protein (IN). Retroviral integration occurs in 2 catalytic steps, referred to as 3'-processing and strand transfer. 3'-processing takes place in the cytoplasm within the preintegration complex. During this step, integrase removes a pGT dinucleotide at each 3'-end of the viral long terminal repeats (LTRs) which exposes a 3'-OH group of an adjacent highly conserved cytosine-adenosine (C-A) dinucleotide. The following strand transfer occurs in the nucleus where IN mediates a concerted nucleophilic attack by each of the 3'-hydroxyl residues of the viral DNA on the host target DNA. In this reaction the viral DNA ends are ligated into the target DNA and the process is completed by cleavage of the unpaired dinucleotides from the 5'-ends of the viral DNA. The single stranded gaps created in this process are most likely repaired by host-cell DNA-repair enzymes (Figure 1.9; reviewed in Van Maele *et al.*, 2006).

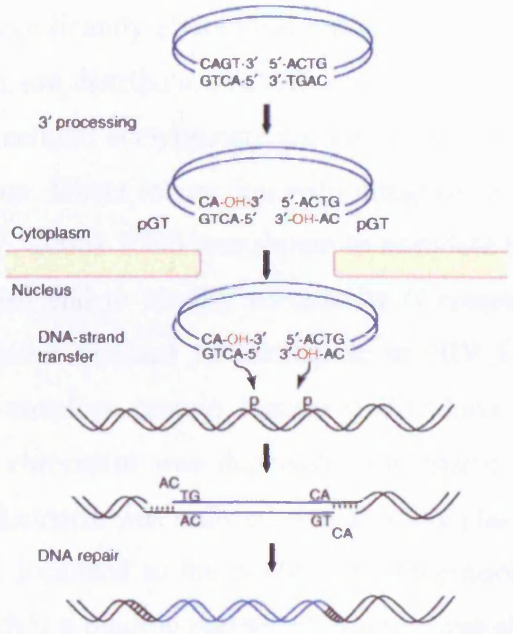


Figure 1.9. Outline of the integration reaction. The 3'-processing reaction occurs in the cytoplasm, whereas DNA-strand transfer takes place in the nucleus. The strand-transfer reaction is concerted: both viral DNA ends are inserted into the host chromosomal DNA at the same time. In the case of HIV-1, the distance between the integration sites of both ends is always five base pairs. Repair of the remaining gaps in the chromosomal DNA results in a five-base-pair duplication of the host cell genome. Van Maele *et al.*, 2006.

Several host factors have been proposed to be important in the course of HIV-1 integration. The integrase interactor 1 (Ini1 or hSNF5), a subunit of the SWI/SNF chromatin-remodelling complex, has been shown to stimulate the *in vitro* DNA-joining

activity of IN (Kalpana *et al.*, 1994). The high mobility group chromosomal protein A1 (HMG A1), a non-histone DNA binding protein important for transcriptional regulation and chromatin structure, has also been shown to stimulate the integration reaction *in vitro*. This effect may be regulated through induced changes in DNA structure (Farnet and Bushman, 1997). The barrier-to-autointegration factor (BAF), a small basic DNA binding protein, not matching any other identified protein, prevents intramolecular integration of the viral cDNA, possibly through condensing the viral DNA in the PIC (Lee and Craigie, 1998; Zheng *et al.*, 2000). Lens epithelium-derived growth factor (LEDGF/p75), a protein implicated in the regulation of gene expression and the cellular stress response, was also found to interact with lentiviral IN (Cherepanov *et al.*, 2003) and to be a component of the PIC (Llano *et al.*, 2004b). Its proposed roles in retroviral integration are a) protecting the IN from proteasomal degradation (Llano *et al.*, 2004a) and b) assisting with tethering of IN to the chromosomal DNA thereby increasing the likelihood that viral cDNA will integrate into a transcriptionally active region (Maertens *et al.*, 2003; Ciuffi *et al.*, 2005). A strong knock-down of LEDGF/p75 in cells has been reported to not only significantly affect viral replication (Llano *et al.*, 2006) but also to affect HIV integration site distribution (Ciuffi *et al.*, 2005). Another factor involved in integration is p300, a cellular acetyltransferase known to acetylate histones and regulate chromatin conformation. Direct interaction with integrase was shown both *in vitro* and *in vivo* (Cereseto *et al.*, 2005). P300 was shown to acetylate three specific lysines in the C-terminus of integrase and to modify its activity (Cereseto *et al.*, 2005). The most recently discovered factor claimed to participate in HIV-1 integration is emerin, an integral inner-nuclear-envelope protein. Recent studies have shown that the interaction of viral cDNA with chromatin was dependent on emerin and infection of primary macrophages in which emerin was reduced was abortive (Jacque and Stevenson, 2006). Although viral cDNA localised to the nucleus its integration into host chromatin was inefficient. BAF, which is a binding partner of emerin, was shown to be required for the ability of emerin to support HIV-1 replication. However, a later study in which emerin expression was potently reduced in human cells or completely knocked-out in mouse cells, failed to confirm the importance of this factor in HIV-1 infectivity (Shun *et al.*, 2007).

Mapping over 500 integration events of HIV-1 and HIV-1-derived retroviral vectors in a human T-cell line revealed that integration preferentially occurs in genes highly transcribed by RNA polymerase II, without a preference for introns or exons (Schroder

et al., 2002). MLV, by contrast, favours integration at or near gene promoter regions (Mitchell *et al.*, 2004; Ciuffi and Bushman, 2006).

1.2.3.7 The late phase of HIV infection

The late phase of the retroviral life cycle begins with the synthesis of viral RNAs and proteins, continues with the assembly of virus particles and ends with the budding of progeny virions.

Once integrated, the provirus serves as a template for the transcription of viral genes. Transcription is carried out by RNA polymerase II which recognises a promoter region located in the 5' LTR. The LTR also possesses binding sites for numerous cellular transcription factors, including NF-κB, Sp1, AP-1 and NF-AT which are important in controlling viral gene expression (reviewed in Pereira *et al.*, 2000; Freed, 2004; Wu, 2004; Richter and Palu, 2006). Despite the importance of these factors, transcription initiated from the HIV-1 promoter proceeds inefficiently and requires the viral protein Tat to enhance the processivity of the RNA polymerase II complex.

Unlike most transcriptional activators, Tat mediates trans-activation by interacting with RNA rather than with DNA (Berkhout *et al.*, 1989). This interaction occurs specifically between Tat and a specific 59-residue RNA hairpin at the 5' end of nascent viral transcripts known as the TAR (trans-activating response element; see chapter 1.2.2). Tat binds to the TAR as a complex with the human cellular factor cyclin T1, which leads to recruitment of cyclin-dependent kinase 9 (Cdk9) to the TAR element (Herrmann and Rice, 1993; Wei *et al.*, 1998). Kinase activities of Cdk9 are activated by this recruitment and result in the hyper-phosphorylation of the C-terminal domain of RNA polymerase II, thereby stimulating transcriptional elongation (Price, 2000).

Successful transcription of the HIV provirus produces a single full-length primary transcript, which in some cases is partially or fully spliced to produce mRNA for HIV proteins and in some cases is left unspliced. Unspliced RNA transcripts serve as the genomic RNA and are encapsidated into progeny virions. Unspliced RNA also acts as a template for the synthesis of Gag and Gag-Pol gene products. Because nuclear export of unspliced/partially spliced, intron containing, cellular transcripts is restricted in mammalian cells, HIV has evolved the viral Rev protein to overcome this obstacle. Rev binds to a *cis*-acting RNA element called the Rev-response element (RRE) present in the 3' region of all unspliced or partially spliced HIV transcripts (Malim *et al.*, 1989; reviewed in Cullen, 2003; Freed, 2004). Nuclear export of RNA-Rev complexes

involves a direct interaction of Rev with the nucleocytoplasmic transport factor Crm1 (chromosome region maintenance 1), in conjunction with a GTP-bound form of Ran GTPase (Fornerod *et al.*, 1997). Once the RNA-Rev-Crm1-Ran-GTP complex has translocated through nuclear pores to the cytoplasm, the Ran-bound GTP is hydrolyzed to GDP and the complex disassembles. Recently a new protein called DDX3 was shown to be involved in this process. DDX3 is a member of the DEAD box RNA helicases located around nuclear pores that bind Crm1 (Yedavalli *et al.*, 2004). Rev is imported back to the nucleus by its association with the cellular nuclear import receptor importin β.

Once within the cytosol, viral mRNA is translated into proteins by the host cell translation machinery (reviewed in Coffin *et al.*, 1997; Goff, 2001a). Structural proteins encoded by the Gag gene are translated into a 55 kDa polyprotein that contains MA, CA, NC and p6 proteins. The full-length unprocessed mRNA, identical in sequence to the genomic RNA, serves as a template for the translation. Sometimes, in about 10% of Gag translation events, the cellular machinery undergoes a -1 frameshift in the p6 coding region and allows reading through the *gag* stop codon to the adjacent *pol* reading frame. This results in the formation of a 160 kDa Gag-Pol fusion protein which, apart from the structural proteins, also contains PR, RT and IN. These individual protein components are later released by the activity of viral protease. While Gag and Gag-Pol polyproteins are synthesised by cytoplasmic ribosomes, *env* mRNA, encoding the envelope polyprotein gp160, is translated on rough endoplasmic reticulum-associated ribosomes. Inside the endoplasmic reticulum gp160 is glycosylated, associated into trimers and cleaved by cellular furin proteases into its SU and TM subunits. This complex of envelope proteins is then trafficked through the Golgi to the cell membrane for virion assembly and budding.

Encapsidation of full-length viral genomic RNA is specific and occurs in the cytoplasm. Specificity is mediated by *cis*-acting packaging signals, which are mainly located in the 5' untranslated leader region in the genomic RNA, and by viral proteins acting in *trans*, especially the viral Gag polyprotein. Although the components involved in packaging are similar between HIV-1 and HIV-2, the mechanisms appear to be slightly different (Kaye and Lever, 1999). The core packaging sequence in HIV-1 is situated immediately downstream of the major splice donor (Lever *et al.*, 1989), and thus is present only on the viral genomic RNA. The HIV-1 Gag polyprotein specifically recognises and binds to RNAs that contains this encapsidation signal (ψ) (Kaye and Lever, 1996) and is able to encapsidate RNA without being translated in *cis* from the viral genome (McBride *et*

al., 1997). In contrast, HIV-2 contains its packaging region upstream of the splice donor (McCann and Lever, 1997; Griffin *et al.*, 2001) and thus all viral messages contain the encapsidation signal. HIV-2 encapsidates its genomic RNA cotranslationally, so that only genomic HIV-2 RNAs which are templates for a full-length Gag polyprotein are efficiently incorporated into progeny virions (Kaye and Lever, 1999). This mechanism has been termed *cis*-acting encapsidation and results in a non-reciprocal packaging relationship between HIV-1 and HIV-2. Whilst wild-type HIV-2 is able to encapsidate mainly its own RNA, HIV-1 efficiently encapsidates both HIV-1 and HIV-2 vector constructs in addition to its own RNA (Kaye and Lever, 1998).

Expression of the retroviral Gag polyprotein by itself is sufficient for the formation of virion-like particles (Karacostas *et al.*, 1989; Morikawa *et al.*, 1999). The HIV-1 Gag polyprotein contains three important domains which play roles in assembly and budding. They are the membrane targeting (M), interaction (I), and late (L) domains (Wills and Craven, 1991). The M-domain is located within the MA region. It is modified by a covalent addition of myristate to its first glycine residue which is followed by a stretch of basic residues. Both of these signals are required for membrane-targeting function of Gag (Bryant *et al.*, 1989; Gottlinger *et al.*, 1989; Martin-Serrano and Bieniasz, 2003a; Lee and Linial, 1994). The I-domain is responsible for Gag multimerisation and is located mainly in the NC region (Sandefur *et al.*, 2000). Retroviral budding is mediated by the L-domain through the conserved P(T/S)AP and LYPLxxL motifs located in the p6 region of HIV-1 Gag (Gottlinger *et al.*, 1991; Huang *et al.*, 1995; Strack *et al.*, 2003). In the case of MLV a similar L-domain motif is present within the p12 viral protein (Yuan *et al.*, 1999). The L-domain was shown to require modification by mono-ubiquitylation to mediate budding (Ott *et al.*, 1998; Patnaik *et al.*, 2000). Recently, the ubiquitin-protein ligase hPOSH was found to participate in this process (Alroy *et al.*, 2005).

A better understanding of the HIV budding process was achieved by the identification of two cellular factors able to interact with the motifs present in the L-domain of p6. Tsg101 was shown to interact with the P(T/S)AP motif and AIP1/Alix was shown to interact with the LYPLxxL motif (Garrus *et al.*, 2001; Verplank *et al.*, 2001; Strack *et al.*, 2003). These findings indicated the connection between the endosomal sorting pathway and HIV-1 budding. Tsg101 and AIP1/Alix are both members of the Class E vacuolar sorting machinery (Vps). Tsg101 is a component of the ESCRT-I (endosomal sorting complex required for transport-1) complex and AIP1/Alix appears to serve as a connector between the ESCRT-I and ESCRT-III sorting pathways by simultaneously

interacting with Tsg101 and CHMP (a member of ESCRT-III complex) proteins (Figure 1.10; Martin-Serrano *et al.*, 2003b). Altogether, there are three ESCRT multiprotein complexes (termed as ESCRT-I, II, III) present in mammalian cells whose function is to sort cargo proteins in the multivesicular body (MVB) pathway. Proteins can enter multivesicular bodies either via endocytosis from the plasma membrane or via vesicular trafficking from the Golgi (reviewed in Cimarelli and Darlix, 2002; Demirow and Freed, 2004; Gomez and Hope, 2005). HIV and other viruses can use these components, normally involved in the intracellular sorting of membrane proteins, to exit infected cells. HIV-1 assembly and budding takes place in the lipid raft regions of the plasma membrane (Nguyen and Hildreth, 2000). Some studies, examining HIV-1 assembly and budding in macrophages, reported that it can also occur in MVBs and that the virions then exit cells using the pre-existing exosome pathway (Nguyen *et al.*, 2003). However, a recent study disagreed with these findings and showed that it is assembly at plasma membrane that is essential for virus release and the endosomal localisation of HIV-1 Gag and virions occurs as a result of their internalisation from the plasma membrane (Jouvenet *et al.*, 2006).

As shown in Figure 1.10, HIV assembly and budding starts with the targeting of Gag to the membrane together with viral RNA and additional viral proteins, which is followed by the multimerisation of Gags and recruitment of the ESCRT system through Tsg101, AIP1/Alix and other cellular proteins which allows the membrane deformation and later fission. Maturation of the virion particle occurs during or after it buds from the host cell. During maturation, the HIV protease cleaves the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion.

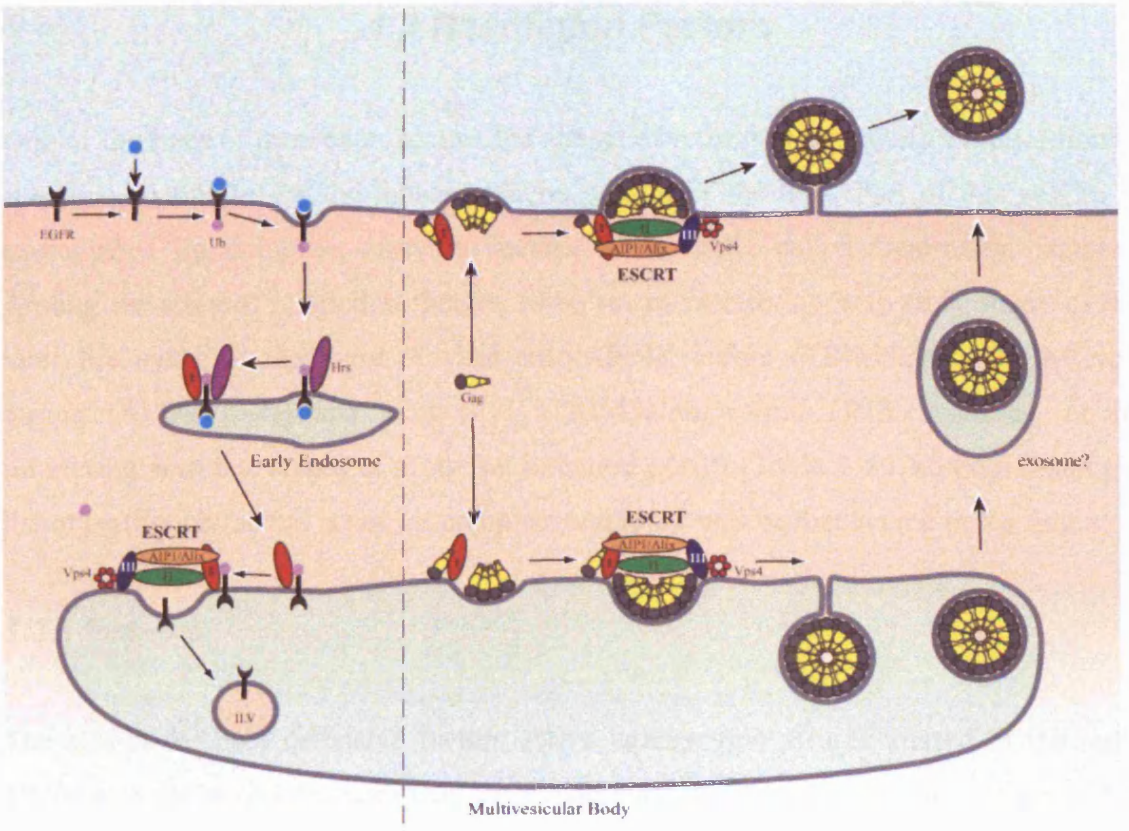


Figure 1.10. A model for retrovirus release. On the left is a schematic representation of endocytosis and MVB sorting of an activated growth factor receptor. On the right is depicted the hijacking of MVB sorting machinery for virus release. HIV-1 Gag is believed to mimic Hrs in triggering membrane recruitment of ESCRT-I. Virus particles are shown to assemble and bud at the plasma membrane or to be released (e.g., from macrophages) through the exosome pathway following assembly in the MVB. Additional details provided in the text. Demirow and Freed 2004.

1.3 Restriction Factors

One of the lines of protection against the spread of retroviral infections within different species is conferred by the innate immune system of the host. Part of this system is exemplified by dominant antiviral factors within cells called "restriction factors". Among the array of restriction factors, some act by interfering with early stages of the viral life cycle; at the point of viral entry (Fv4), before (TRIM5alpha, TRIMCyp), during (APOBEC3G) and after (Fv1, TRIM5alpha) viral DNA synthesis, or by interfering with late stages of retroviral infection (ZAP) (Table 1.4). Without doubt the list of restriction factors is not yet complete and more will be discovered in the future.

1.3.1 Fv4

The search for host defensive factors active against retroviruses started in the early 1970s with the study of viruses that cause malignancies in mice. A number of genes that affect the susceptibility of mice to infection by Murine leukaemia virus were soon described (Lilly and Pincus, 1973). One of these genes, *Fv4*, located on chromosome 12, is able to block exogenous infection with ecotropic Friend murine leukaemia virus *in vivo*, *in vitro* and in transgenic mice expressing the cloned Fv4 gene (Kai *et al.*, 1976; Odaka *et al.*, 1981; Ikeda and Sugimura, 1989; Limjoco *et al.*, 1993). Fv4 was shown to be expressed at high levels in mouse cells and molecular cloning revealed that it encoded a defective ecotropic MLV-related provirus lacking *gag* and most of the *pol* sequence but retaining a complete *env* gene (Kozak *et al.*, 1984; Ikeda *et al.*, 1985). Nucleotide sequencing revealed over 70% homology between Fv4 and ecotropic MLV *env* genes and several substitution mutations were present which when introduced into the wild-type MLV *env* gene blocked viral replication. A viral interference model has been proposed as the mechanism of Fv4 restriction (Ikeda *et al.*, 1985; Kai *et al.*, 1986). Virus interference refers to the phenomenon in which cells chronically infected with one virus are resistant to superinfection with other viruses bearing the same virus envelope specificity. Interference is due to the blockade of the virus receptors by the envelope glycoprotein of the infecting virus or of an endogenous provirus.

Species	Restriction factor	Time of Block	Restricted viruses	Unrestricted viruses
Mouse	Fv4	Entry	Ecotropic MLV	Amphotropic MLV, lentiviruses
Mouse (NIH Swiss)	$Fv1^n$	Post-reverse transcription, before integration	B-MLV	N-MLV, NB-MLV, HIV-1
Mouse (Balb/c)	$Fv1^b$	Post-reverse transcription before integration	N-MLV	B-MLV, NB-MLV
Human	TRIM5α/Ref1	Early post-entry before reverse transcription + post-reverse transcription	N-MLV, EIAV, FIV, moderately HIV-1	B-MLV, NB-MLV, HIV-2, SIV_{mac}, SIV_{Agm}
Human	APOBEC3G	Viral DNA formation	Vif-negative HIV-1, SIV_{Agm}, EIAV, HBV	Vif-positive HIV, MLV
Cow	TRIM505265/ Bovine Lv1	before reverse transcription, some cases after reverse transcription	N-MLV, HIV-1, HIV-2, EIAV, FIV	B-MLV, NB-MLV
Rat	ZAP	Viral RNA expression	MLV, alphaviruses	VSV, poliovirus
Old World Monkeys				
Chimpanzee	TRIM5α	?	N-MLV, moderately HIV-1	SIV_{mac}, SIV_{Agm}, B-MLV, NB-MLV
Orangutan	TRIM5α	?	N-MLV, moderately HIV-1	SIV_{mac}, SIV_{Agm}, B-MLV, NB-MLV
Rhesus macaque	TRIM5α/Lv1	Early post-entry before reverse transcription+ post-reverse transcription	HIV-1, FIV, SIV_{Agm}	N-MLV, B-MLV, SIV_{mac}
Cynomolgus monkey	TRIM5α	?	HIV-1	SIV_{mac}
African green monkey/*tantalus*	TRIM5α/Lv1	Early post-entry before reverse transcription	N-MLV, HIV-1, HIV-2, SIV_{mac}, FIV, EIAV	B-MLV, SIV_{Agm}
African green monkey/*pygerythrus*	TRIM5α	?	N-MLV, HIV-1	SIV_{mac}, NB-MLV, SIV_{Agm}, B-MLV
New World Monkeys				
Squirrel monkey	TRIM5α/Lv1	Post-entry after reverse transcription	SIV_{mac}, moderately SIV_{Agm}	N-MLV, B-MLV, HIV-1, NB-MLV
Owl monkey	TRIMCyp	Early post-entry before reverse transcription+ post-reverse transcription	HIV-1	N-MLV, B-MLV, SIV_{mac}
Tamarin	TRIM5α	?	SIV_{mac}, moderately HIV-1, SIV_{Agm} and N-MLV	B-MLV, NB-MLV
Spider monkey	TRIM5α	?	SIV_{mac}, SIV_{Agm}, HIV-1, moderately N-MLV	B-MLV, NB-MLV

Table 1.4. Properties of dominant resistance genes. Lilly, 1967; Hofmann *et al.*, 1999; Towers *et al.*, 2000; Besnier *et al.*, 2002; Cowan *et al.*, 2002; Besnier *et al.*, 2003; Hatziioannou *et al.*, 2003; Owens *et al.*, 2003; Hatziioannou *et al.*, 2004b; Keckesova *et al.*, 2004; Nisole *et al.*, 2004; Perron *et al.*, 2004; Sayah *et al.*, 2004b; Stremlau *et al.*, 2004; Yap *et al.*, 2004; Nakayama *et al.*, 2005; Saenz *et al.*, 2005; Song *et al.*, 2005c; Anderson *et al.*, 2006; Si *et al.*, 2006; Wu *et al.*, 2006; Ylinen *et al.*, 2006. Abbreviations explained on page 12.

1.3.2 Fv1

Another example of a gene encoding a restriction factor that inhibits the replication of Murine leukaemia retroviruses in mice is Fv1 (Lilly *et al.*, 1967). Fv1 (Friend virus susceptibility 1) gene has three major restricting alleles among inbred strains of mice. The $Fv1^n$ allele, found in NIH Swiss mice, allows replication of N-tropic MLV (N-MLV) strains of virus and blocks very closely related B-tropic strains (B-MLV). The $Fv1^b$ allele, present in Balb/c mice, shows precisely the opposite phenotype; allowing replication of B-MLV and blocking N-MLV. A third allele, called $Fv1^{nr}$ inhibits infection by B-MLV and some N-MLV strains. Some strains of MLV, termed NB-tropic (NB-MLV), efficiently infect mouse cells irrespective of the presence of any of these alleles. The vast majority of divergent mice species encode full length Fv1 alleles (Qi *et al.*, 1998) but most of them do not restrict the narrow range of mouse viruses tested thus far. The Fv1 alleles from mice, which are equivalently susceptible to infection by N-, B-, and NB-tropic MLV strains, have been referred to as a null Fv1 alleles ($Fv1^0$) (Kozak, 1985). Fv1 was shown to be co-dominant, so that $Fv1^{n/b}$ heterozygous animals are resistant to both N-, and B-MLV (Hartley *et al.*, 1970; Pincus *et al.*, 1971; Hartley and Rowe, 1975).

Construction of viral DNA recombinants between N- and B-tropic MLVs mapped the viral determinant for N and B tropism to the capsid (CA) protein of the virus (DesGroseillers and Jolicoeur, 1983). It was shown to be at position 110, with an arginine specifying N-tropism and a glutamate B-tropism. Substitutions of glutamate with another acidic amino-acid, like aspartate, also produced B-tropic virus and substitution of arginine with another basic amino-acid such as lysine kept the N-tropism of the virus. This suggested that to change N to B a charge change from positive to negative must take place (Kozak and Chakraborti, 1996).

Analyses of viral DNA in infected cells showed that Fv1 blocks virus infection in the early stages of infection, after reverse transcription, but before the entry of the virus into the nucleus. This is indicated by similar levels of linear DNA in restricted versus unrestricted infections but a lower accumulation of circular viral DNA, which is thought to be indicative of viral nuclear entry, in restricted infection (Jolicoeur and Rassart, 1980; Yang *et al.*, 1980). Furthermore, functional PICs can be isolated from Fv1-restrictive cells (Pryciak and Varmus, 1992). Fv1 restriction is not dependent on a specific route of entry because sensitive virus is restricted whether it enters cells via

amphotropic, ecotropic or vesicular stomatitis virus envelopes, which are known to utilise different routes of entry.

The Fv1 gene was isolated by a positional cloning strategy (Best *et al.*, 1996). It encodes a protein that exhibits 43% and 60% homology at the nucleotide level to the *gag* gene of the ERV-L family of endogenous retroviral elements in mice and humans, respectively (Best *et al.*, 1996; Benit *et al.*, 1997). So far no other ERV-L-like elements have been shown to contribute to viral permissivity, although there are many that are even more closely related to MLV than Fv1 (Benit *et al.*, 1997). Examination of DNA, by Southern blot, from variety of mammals (mouse, cat, rat, bat, human, pig, cow, African green monkey, hamster) revealed that the Fv1 gene is unique to mice (Best *et al.*, 1996; Besnier *et al.*, 2003). Furthermore, Fv1 alleles active against MLV are found only in mouse strains that contain many endogenous MLVs suggesting that antiviral activity probably evolved in response to the presence of these viruses. The N and B alleles of Fv1 differ at two internal amino-acid positions (358 and 399) and at the C-terminus (Best *et al.*, 1996). Fv1 is one of the examples of how defective retroviral DNAs carried in the genome can sometimes be useful for the host. The Gag origin of Fv1 is also supported by the presence of a highly conserved sequence known as the major homology region (MHR) (Benit *et al.*, 1997). This motif (Q-X3-E-X7-R), whose precise role is unknown, is present in the capsid protein of all retroviruses and has been shown to be important for the viability of retroviruses in both the early and late stages of their life cycle (Mammano *et al.*, 1994; Craven *et al.*, 1995). In Fv1, as well as in the capsid proteins of all other retroviruses, the integrity of this region is required for function, and mutation of these conserved amino-acids in Fv1 abolishes its ability to restrict, suggesting that it is functioning in a CA-like manner (Bishop *et al.*, 2001).

Retroviral capsid proteins are known to multimerise and the simplest model for Fv1 action is that it can bind to the capsid protein of incoming viral preintegration complexes and blocks their proper progression into the nucleus for DNA integration. (Best *et al.*, 1996; Goff, 1996). This notion is consistent with the observation that a sufficiently large dose of incoming sensitive Gag can titre out the Fv1 product and allow the remaining virus to escape the restriction. The block to infection is then said to be abrogated. Later it was shown that abrogating virus must be introduced into the cell as an incoming virion particle because expression of Gag or CA proteins does not abrogate restriction of incoming virus (Bassin *et al.*, 1980; Duran-Troise *et al.*, 1981; Dodding *et al.*, 2005). However, no form of physical interaction between Fv1 and CA protein of the virus has been demonstrated so far and the mechanism of Fv1 restriction

remains unclear. A recent report demonstrated that the MLV CA is sumoylated and suggested this modification was necessary for trafficking of the PIC to the nucleus. Perhaps Fv1 blocks sumoylation of the capsid thereby trapping the PIC in a non-infectious cellular compartment (Yueh *et al.*, 2006).

By using microscopy, Fv1 was found to be expressed at extremely low levels and to be localised in the cytoplasm where it interacts with the tubules of the Trans Golgi network (Yap and Stoye, 2003). A recent report suggests that Fv1 might act as a homo-dimer (Bishop *et al.*, 2006b).

1.3.3 Ref1/Lv1

Although Southern blot and genome sequence analysis indicates that Fv1 is unique to mouse, many human and primate cell lines were also shown to restrict infection by N-MLV and other retroviruses (Table 1.4; Towers *et al.*, 2000, 2002; Besnier *et al.*, 2002; Hatziioannou *et al.*, 2003). A number of human cell lines exhibited resistance to vesicular stomatitis (VSV-G)-pseudotyped N-MLV, to an equine infectious anaemia virus (EIAV), a lentivirus that naturally infects horses, and moderately to HIV-2, but not to B-MLV or any other primate lentiviruses tested so far. Most Old World monkey cell lines (African and Asian; for example macaque and African green monkey) restrict infection by VSV-G-pseudotyped HIV-1, while the majority of the New World monkey cell lines (South American for example Squirrel monkey) restrict infection by SIV_{mac} (Hofmann *et al.*, 1999). Several monkey species restrict a broader range of retroviruses. African green monkey cells restrict macaque simian immunodeficiency virus (SIV_{mac}), HIV-1, HIV-2, N-MLV and EIAV. The putative factors responsible for this restriction, named restriction factor-1 (Ref1) in humans and lentivirus susceptibility factor 1 (Lv1) in simians, share several key attributes that are characteristic for Fv1 (Besnier *et al.*, 2002; Cowan *et al.*, 2002; Munk *et al.*, 2002; Towers *et al.*, 2002). The resistant phenotype is dominant; fusion of HIV-1-permissive human cells with HIV-1-resistant monkey cells results in heterokaryons that are HIV-1 resistant (Cowan *et al.*, 2002; Munk *et al.*, 2002). This suggested the presence of an inhibitor in monkeys and not merely the lack of a host factor necessary for the virus life cycle. Moreover, restriction of sensitive viruses could be abrogated by preexposure of cells to restricted virus or virus-like particles (VLP). Exposure of human cells to a high dose of N-MLV virus could abrogate restriction to a second dose of N-tropic virus and preexposure of Owl monkey or Rhesus macaque cells to high dose of HIV-1 increased the titre of a second

HIV-1 dose (Besnier *et al.*, 2002; Cowan *et al.*, 2002; Munk *et al.*, 2002; Towers *et al.*, 2002; Kootstra *et al.*, 2003). By using a chimeric HIV-1/SIV_{mac} virus it was demonstrated that the viral determinant is in the capsid protein. Replacement of the capsid or parts of capsid in SIV_{mac} with that of HIV-1 resulted in a virus that behaves largely, in terms of its tropism for multiple simian cell lines, as if it was HIV-1 rather than SIV_{mac} (Dorfman and Gottlinger, 1996; Cowan *et al.*, 2002; Hatziioannou *et al.*, 2003, 2004a; Owens *et al.*, 2003). In the case of MLV, the same amino-acid 110 within CA that differentiates between N- and B-tropism in mice also determines the tropism for human and monkey cells (Towers *et al.*, 2000). Abrogation requires Gag cleavage, suggesting that the interacting domain on CA is masked on the precursor protein, and implying that the interaction can only occur with mature virion cores but not with the immature virions which exit the cell (Dodding *et al.*, 2005). This also provides an explanation for the observation that MLV producing cells are fully Fv1 protected. Restriction does not depend on the route of viral entry into the cell. HIV-1 virions which expressed wild-type envelope on their surfaces and fused at the plasma membrane were just as restricted as HIV-1 virions pseudotyped with the VSV-G envelope which entered the cell through the endocytic pathway. The only obvious difference in the phenotypes conferred by Fv1 and Ref1/Lv1 is that Fv1 acts primarily after the completion of reverse transcription, while Ref1 and Lv1 usually block the virus before reverse transcription as documented by studies of viral DNA formation (Shibata *et al.*, 1995; Towers *et al.*, 2000; Cowan *et al.*, 2002).

Other mammalian species, such as cows, pigs and bats, also specifically restrict N-MLV, implying that retrovirus restriction factors in mammals are widespread (Besnier *et al.*, 2003). The ability of cells from African green monkeys to restrict both, C-type retroviruses and lentiviruses allowed the investigation of whether these distinct retroviruses whose capsids share little sequence homology are inhibited by the same restriction factor. Cross abrogation experiments showed that exposure of Agm cells individually to all restricted viruses, but not unrestricted B-MLV, could saturate restriction to N-MLV. Similarly, in human cells, treatment with restricted EIAV VLPs is able to restore susceptibility to N-MLV. These results strongly implied that a common factor is able to recognise multiple divergent retroviruses. (Besnier *et al.*, 2002; Hatziioannou *et al.*, 2003).

1.3.4 TRIM5α

Long-standing efforts to find an antiviral factor responsible for restricting HIV-1 infection in monkey cells bore fruit in early 2004. Stremlau and colleagues used a genetic screen to demonstrate that the tripartite motif (TRIM) containing protein TRIM5α from Rhesus monkey, but not human, has the ability to potently restrict HIV-1 replication (Stremlau *et al.*, 2004; reviewed in Goff, 2004; Nisole *et al.*, 2005; Towers, 2005, 2006; Perez and Hope, 2006). Immediately after this discovery the restriction field became very competitive and publications from several laboratories quickly confirmed and extended the importance of TRIM5α as a restriction factor, showing that human and monkey TRIM5α alleles account for the poor species-specific retroviral infectivity described as Ref1 in human and Lv1 in simian cells. Human TRIM5α was shown to be responsible for N-MLV activity (Hatziioannou *et al.*, 2004b; Keckesova *et al.*, 2004; Perron *et al.*, 2004; Yap *et al.*, 2004) and the TRIM5α of African green monkey was shown to have restrictive activity against HIV-1, SIV_{mac}, EIAV and N-MLV (Hatziioannou *et al.*, 2004b; Keckesova *et al.*, 2004; Yap *et al.*, 2004). Activity of human TRIM5α against N-MLV, but not very closely related B-MLV, and discriminating between them on the basis of residue 110 of the capsid protein, suggested a viral determinant for restriction in the capsid protein of sensitive virus (Perron *et al.*, 2004). Interaction of sensitive virus with human TRIM5α was shown to lead to a block in viral reverse transcription (Keckesova *et al.*, 2004; Perron *et al.*, 2004). This was later shown not to be the case in squirrel monkeys. Squirrel monkey TRIM5α, active against SIV_{mac}, does not block SIV_{mac} DNA synthesis (Ylinen *et al.*, 2005). Subsequently, TRIM5α was shown to be responsible for restriction in various Old World and New World primates (Table 1.4; Song *et al.*, 2005c). Interests in other mammals apart from humans and monkeys led to the recent identification of a broadly effective antiviral TRIM protein in cattle, indicating that innate immune activity of TRIM proteins extends beyond primates (Si *et al.*, 2006; Ylinen *et al.*, 2006).

Almost in parallel with the discovery of TRIM5α, a TRIM5-derived restriction factor was identified in Owl monkey cells (OMK). Owl monkeys are atypical New World monkeys because of their ability to inhibit HIV-1 but not SIV_{mac}. However, this block to HIV-1 replication is abrogated when the binding between the capsid protein of HIV-1 and host cyclophilin A is interrupted (Towers *et al.*, 2003). Remarkably, this observation has been explained by the identification of Owl monkey TRIM5 as a fusion protein between TRIM5 and cyclophilin A (TRIMCyp) (Nisole *et al.*, 2004; Sayah *et*

al., 2004b). It appears that a complete *CypA* pseudogene had been inserted between exons 7 and 8 of the TRIM5 gene, thus replacing the C-terminal domain. (Figure 1.11).

1.3.4.1 Main features of TRIM family

Tripartite motif (TRIM) proteins are involved in a wide variety of biological processes ranging from apoptosis through cell cycle regulation to viral response. There are about 70 family members in most mammalian genomes and about 20 members in invertebrate organisms, such as flies and worms.

The tripartite RBCC motif that defines the TRIM proteins includes a RING domain, either one or two B boxes (B1 and B2), and a coiled coil domain (reviewed in detail in Meroni and Diez-Roux, 2005).

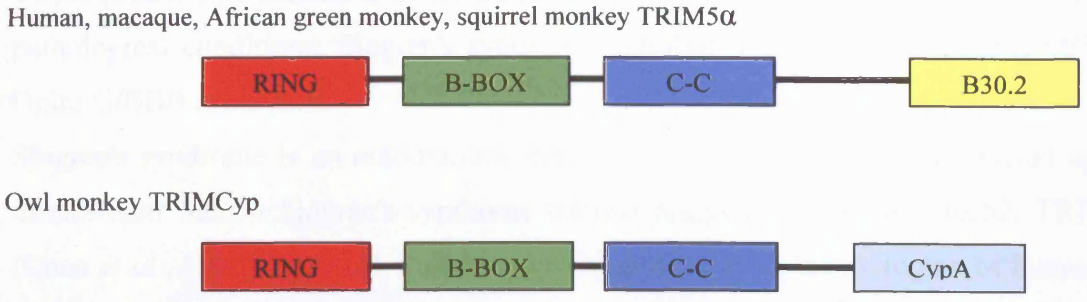


Figure 1.11. Schematic presentation of TRIM5α and TRIMCyp molecules.

The RING domain is a cysteine-rich zinc binding sequence, typically involved in specific protein-protein interactions. Many RING domains have E3-ubiquitin ligase activity. The TRIM5 RING domain was shown to act as a ligase for auto-ubiquitination in *vitro* in the context of the TRIM5δ splice variant (Xu *et al.*, 2003). The B box is a distinct zinc binding motif of around 40 residues putatively involved in protein-protein interactions and present only in TRIM proteins, making it a critical determinant of the TRIM family. The two B-boxes have not only different primary sequence and zinc binding motifs, but also differ in their tertiary structure. The solution of the structure of the B-Box1 from human TRIM18 demonstrated that it binds two zinc atoms and adopts a RING-like fold, raising the possibility that it could either have E3 ligase activity itself or it could enhance and modulate the activity of a nearby RING domain; i.e. confer E4 enzyme activity (Massiah *et al.*, 2006). This was shown not to be the case for a B-Box2 domain which is the only B-Box domain encoded within the TRIM5 gene. The B-Box2

domain binds one zinc atom and its tertiary structure is not similar to any other known zinc-binding motif (Borden *et al.*, 1995). The coiled-coil region is involved in homo- and hetero-dimerisation of TRIM proteins (Reymond *et al.*, 2001).

Some TRIM proteins, including TRIM5α, contain a C-terminal 170 amino-acid long globular B30.2 domain, also found in members of the immunoglobulin superfamily (Henry *et al.*, 1998; Meyer *et al.*, 2003, Rhodes *et al.*, 2005). Although the majority of the TRIM5 literature refers to this domain as a SPRY domain this is in fact incorrect. Whilst SPRY domains are evolutionary ancient, B30.2 domains, found in butyrophilin and TRIM proteins, are more recent domains, comprising the combination of SPRY with an additional, 50-60 residue long, PRY domain. Thus, the B30.2 domain consists of a combination of SPRY and PRY domains (Rhodes *et al.*, 2005). No clear function has been proposed for a B30.2 domain although it is believed to be involved in protein-protein interactions, substrate recognition and/or RNA binding (Ponting *et al.*, 1997). TRIM proteins possessing a B30.2 domain have been implicated in at least 3 different pathological conditions: Sjogren's syndrome, familial Mediterranean fever (FMF) and Opitz G/BBB syndrome.

Sjogren's syndrome is an autoimmune disease where autoantibodies are raised against intracellular 52kDa Sjogren's syndrome nuclear antigen A (SSA/Ro, Ro52, TRIM21) (Chan *et al.*, 1991). TRIM21 was shown to bind directly to the Fc region of human IgG isotypes through its B30.2 domain suggesting a role of TRIM21 in regulation of immunoglobulin G functions (Yang *et al.*, 2000; Rhodes and Trowsdale, 2007).

FMF is an autosomal recessive rheumatic disease characterised by recurrent attacks of fever with peritoneal inflammation. It is caused by mutations in the B30.2 domain of pyrin/marenostrin protein (TRIM20, French FMF Consortium 1997; International FMF Consortium 1997). FMF primarily affects people of a Mediterranean origin. Because of very high frequencies and selective pressures of FMF alleles in this population it has been suggested that it might confer increased resistance to an endemic Mediterranean pathogen (Stoffman *et al.*, 2000).

Opitz G/BBB syndrome is an X-linked inheritance disorder characterised by a developmental midline defect caused by insertions or partial deletions in the B30.2 domain of TRIM18 (MID1) (Quaderi *et al.*, 1997). Mutated TRIM18 is severely affected in its ability to bind microtubules and this results in reduced proteolysis of microtubule-associated protein phophatase 2A and anomalous microtubule dynamics (Trockenbacher *et al.*, 2001).

Human TRIM genes are dispersed throughout the genome with the exception of two clusters; one is located in the major histocompatibility complex (MHC) region on chromosome 6 (TRIM 10, 15, 26, 27, 31, 38, 39, 40) and the other is located on chromosome 11 (TRIM 5, 6, 21, 22, 34) (Reymond *et al.*, 2001; Meyer *et al.*, 2003). Each of the various TRIM proteins is localised in particular cellular compartments with TRIM5α being present in cytoplasmic bodies when expressed exogenously (Reymond *et al.*, 2001). The role of cytoplasmic bodies is further discussed in chapter 1.3.4.5.

The most famous and well known TRIM protein is the promyelocytic leukaemia (PML, TRIM 19) protein, first identified as a fusion partner with the retinoic acid receptor in human acute promyelocytic leukaemia (de The *et al.*, 1991). PML is localised in matrix-associated multi-protein complexes within nuclei, known as PML nuclear bodies (Dyck *et al.*, 1994). PML is essential for the formation and subsequent function of these bodies which have been implicated in a wide variety of cellular functions, including gene expression, chromatin dynamics, apoptosis, senescence, DNA repair, response to interferon and viral infection. Interest in the interactions between DNA viruses and PML bodies started with the observation of disruption of PML bodies during herpes simplex-1 (HSV-1) infection (Maul *et al.*, 1993). After the entry of the HSV-1 genome into the nucleus PML bodies associate with the viral nucleoprotein complexes. This occurs through *de novo* assembly of PML-like bodies at these sites rather than the movement of pre-existing PML bodies towards viral genomes (Everett and Murray, 2005). It is thought that this represents an intrinsic cellular response aimed at repressing viral gene expression. HSV-1 encodes a protein called ICP0 which is responsible for degradation of the PML protein, thus destroying PML bodies and counteracting cellular antiviral activity (Maul *et al.*, 1993). The observation of association with and/or degradation of PML bodies was extended to papovaviruses (SV40 and Polyomavirus), adenoviruses, parvoviruses, other members of *herpesviridae* family and some RNA viruses (Ahn *et al.*, 1998; Chee *et al.*, 2003; Rosa-Calatrava *et al.*, 2003; reviewed in Everett, 2001, 2006; Regad and Chelbi-Alix, 2001).

Apart from TRIM5α and PML, other members of the TRIM family have been implicated in antiviral processes. The TRIM1 gene from human, African green monkey and owl monkey cells and TRIM22 and TRIM34 from human cells have been reported to weakly restrict N-MLV, HIV-1 and SIV_{mac} respectively (Tissot and Mechti, 1995; Yap *et al.*, 2004; Li *et al.*, 2006a; Zhang *et al.*, 2006). The role of TRIM proteins in innate immunity is supported by the upregulation of some of its members by interferons (IFNs, Regad and Chelbi-Alix, 2001). TRIM5α expression is induced by both type I and

II IFNs and the interferon responsiveness is mainly mediated through an interferon-stimulated response element (ISRE) sequence in the proximal promoter region of TRIM5α (Asaoka *et al.*, 2005).

1.3.4.2 Involvement of individual TRIM5α domains in retroviral restriction

Differential splicing of TRIM5 transcripts results in the production of different TRIM5 isoforms each increasingly shorter from their C-terminus (Figure 1.12; Reymond *et al.*, 2001). Five of them (α, β, γ, δ, ε) have been isolated and three of them (α, γ, δ) have been functionally characterised.

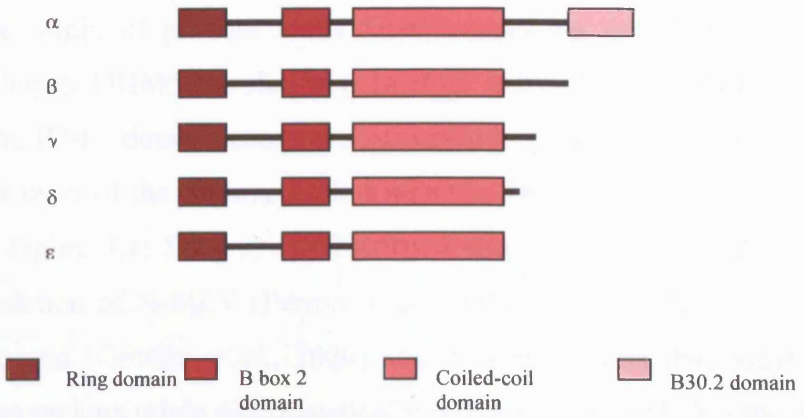


Figure 1.12. Splice variants of human TRIM5 gene. Schematic presentation of different TRIM5 isoforms. Modified from Reymond *et al.*, 2001.

TRIM5α is the longest, the only one to contain a B30.2 domain and the only one to have antiviral activity, indicating the importance of the B30.2 domain for restriction. This was confirmed by mutational analysis of TRIM5α, where a number of groups have exchanged sections between human and simian TRIM5α genes and tested the resulting chimeras for restriction activity (Nakayama *et al.*, 2005; Perez-Caballero *et al.*, 2005a; Sawyer *et al.*, 2005; Stremlau *et al.*, 2005; Yap *et al.*, 2005). These studies showed that it was mainly the B30.2 domain that was responsible for the species-specific properties of TRIM5α. More detailed mapping within the B30.2 domain revealed that a single amino-acid change in the human TRIM5α gene to the corresponding residue in Rhesus macaque TRIM5α (R332P) was sufficient to lead to HIV-1 restriction by the human protein, although not as strongly as the wild-type simian protein (Stremlau *et al.*, 2005; Yap *et al.*, 2005). Surprisingly, this mutant was also able to restrict SIV_{mac}, a property not shared by either of the wild-type TRIM5α proteins (Stremlau *et al.*, 2005). Later investigation revealed that the potentiation of HIV-1 restriction by the R332P mutant of

human TRIM5α results from the removal of the positively charged arginine rather than from introducing the proline residue (Li *et al.*, 2006c). In the case of N-MLV restriction, finding the determinants seems to be more complex and data suggest that apart from the B30.2 domain, the coiled-coil domain is also important for restriction (Yap *et al.*, 2005). As the residues of the B30.2 domain are responsible for virus detection, it is in the best interest of the virus to change its structure, thus evading recognition by TRIM5α. This competition between virus and TRIM5α leads to a so called "Red Queen" effect represented by the rapid replacement of amino-acids at the interaction interface between pathogen and restriction factor. Indeed, analysis of TRIM5α molecules from numerous primate species revealed that the B30.2 domain had undergone intensive positive selection, including many insertions and deletions that predate the origin of primate lentiviruses, suggesting that distinct episodes of viral infection shaped TRIM5α evolution (Liu *et al.*, 2005; Sawyer *et al.*, 2005; Song *et al.*, 2005b). The B30.2 domain contains four variable regions, designated V1 to V4 which account for most of the differences between the TRIM5α alleles from different species (shown in Figure 3.1; Song *et al.*, 2005b). Variable regions 1 and 3 contribute to the potent restriction of N-MLV (Perron *et al.*, 2006b). These regions are predicted to be surface-exposed (Grutter *et al.*, 2006) and it is speculated that some of the residues within these regions might electrostatically interact with N-MLV capsids (Perron *et al.*, 2006b). Recent work investigating the B30.2 domain antiviral specificity determinants showed that, apart from variable region 1 and 3, variable region 2 is also important for restriction of HIV-1 and SIV_{mac} (Ohkura *et al.*, 2006). Fusing the B30.2 domain from one TRIM protein to the RBCC domain of a second TRIM protein can, in most cases, transfer the antiviral specificity of the first to the resulting fusion protein (Yap *et al.*, 2005; Ohkura *et al.*, 2006). Collectively, this supports the importance of the B30.2 domain for the virus-recognition properties of TRIM5α.

It is still unclear whether the RING domain plays a role in restriction or whether it is required for a function unrelated to antiviral activity. When the critical cysteine residues in the RING domain are removed, or when this domain is completely deleted, the resulting TRIM5α mutant is still able to restrict, although with a significantly reduced antiviral activity (Stremlau *et al.*, 2004; Javanbakht *et al.*, 2005). The RING domain of Rhesus TRIM5α was shown to contribute to auto-polyubiquitylation resulting in a rapid, proteasome dependent, turnover of TRIM5 molecules (Diaz-Griffero *et al.*, 2006a). Studies revealed that the half-life of $TRIM5\alpha_{rh}$ is about 60-75 minutes (Diaz-Griffero *et al.*, 2006a; Wu *et al.*, 2006). Exchanging the RING domain between

TRIM5α_{rh} and the more stable TRIM21 extends the half-life of the resulting chimera without having an impact on its anti-HIV-1 restriction properties. Thus, rapid turnover is not an absolute requirement for the ability of TRIM5-related proteins to restrict HIV-1 infection (Diaz-Griffero *et al.*, 2006a). Interestingly, an impaired allele of TRIM5α carrying the RING domain polymorphism (H43Y) has recently been described in the human population (Javanbakht *et al.*, 2006a; Sawyer *et al.*, 2006). The H43Y allele is found at a frequency of 43% in indigenous Central and South Americans. When feline cells over-expressing H43Y TRIM5α, or B-lymphocytes homozygous for this allele, were infected with N-MLV, they were about 100-fold more permissive than cells expressing wild-type TRIM5α. However, the H43Y allele did still retain weak antiviral activity (Javanbakht *et al.*, 2006a; Sawyer *et al.*, 2006). This study supports a role for the RING domain in maximal TRIM5α activity.

The B-box 2 domain appears to be essential for retroviral restriction. Disruption of this domain completely eliminated the anti-retroviral activity of TRIM5α, yet had no effect on TRIM5α multimerisation and capsid binding (Javanbakht *et al.*, 2005; Mische *et al.*, 2005; Perez-Caballero *et al.*, 2005a). These observations suggest that the B-box 2 domain may mediate an effector function critical for retroviral restriction.

TRIM5α proteins from different species exist as trimers, with the coiled-coil domain being necessary for this higher-order formation (Mische *et al.*, 2005; Perez-Caballero *et al.*, 2005a; Javanbakht *et al.*, 2006b). It is proposed that trimerisation allows three B30.2 domains to interact with threefold pseudo-symmetrical structure on the retroviral capsid (Mische *et al.*, 2005). Changes that disrupt TRIM5α trimerisation proportionately affect the ability of TRIM5α to bind retroviral capsid complexes and thus to restrict viral infection (Javanbakht *et al.*, 2006b).

Experiments testing HIV-1 restriction capacities of a series of chimeras between TRIM5α_{rh} and other close human TRIM family members (TRIM6, 21, 34) revealed that in most, but not all, cases heterologous TRIM domains are able to substitute functionally for the RBCC domain of TRIM5α_{rh} (Li *et al.*, 2006b). This was shown not to be the case in restriction of N-MLV, which was much more sensitive to substitution with heterologous RBCC domains (Li *et al.*, 2006b). Additionally, studies where the B30.2 domain of TRIM1, 18, and 19 was replaced by CypA, an HIV-1 capsid binding molecule, showed that it was sufficient to transfer the ability to restrict HIV-1 infection to these heterologous TRIMs that are normally incapable of restricting HIV-1. These experiments supported the functional independence of RBCC and B30.2 domains and

indicated that the overall structure of the RBCC is important for restriction (Yap *et al.*, 2006).

1.3.4.3 A model of the TRIM5α-mediated antiviral mechanism

The exact mechanism by which TRIM5α acts to inhibit retroviral replication is still unclear. Studies have shown that human TRIM5α selectively binds capsid protein from restriction sensitive N-MLV, but not insensitive B-MLV in a B30.2-dependent way (Sebastian and Luban, 2005). This interaction occurs between TRIM5α multimers and capsid multimers only when using the intact virion core, composed of stable viral capsid with properly folded CA amino termini (Shi and Aiken, 2006). Studies of HIV-1 Gag mutants showed that capsid disassembly is a delicate process; both increases and decreases in capsid stability are detrimental to HIV-1 replication (Forshey *et al.*, 2002). Thus, the simplest explanation would be that TRIM5α binding might sequester the incoming virion core and deregulate the controlled uncoating of the sub-viral particles required for proper continuation of viral life-cycle. However, identification of TRIM5 chimeras, which maintained the ability to bind retroviral capsid while losing the ability to restrict, indicated that binding between TRIM5α and the retroviral CA complex is necessary, but not sufficient, for virus-restricting activity (Li *et al.*, 2006b). Thus, upon binding to retroviral capsid, TRIM5α must also exert other effects for the restriction to occur.

Although the presence of a RING domain makes it very tempting to speculate that TRIM5α might act through the ubiquitination or sumoylation of the sub-viral particles followed by their mislocalisation, alteration of conformation or proteasomal degradation the involvement of a RING domain and of the proteasome in TRIM5α-mediated restriction remains controversial. The expression of a restricting TRIM5α in the target cell was shown to correlate with a decrease in the amount of sensitive, particulate viral capsid in the cytosol. In certain cases this loss of particulate CA was accompanied by a detectable increase in soluble capsid protein. These data, combined in some cases, with the use of proteosomal inhibitors, suggested that TRIM5α is promoting a rapid, premature proteasome-independent disassembly of HIV-1 and N-MLV capsid (Perron *et al.*, 2006a; Stremlau *et al.*, 2006a). Similarly, other studies showed that shortly after entry into restrictive cells, HIV-1 CA undergoes an accelerated degradation which is independent of the proteasome (Chatterji *et al.*, 2006). The independence of restriction on the ubiquitin/proteasome system was also documented by experiments, in which

target cells were treated with a proteasome inhibitor or where the ubiquitin-activating (E1) enzyme was inactivated (Perez-Caballero *et al.*, 2005b; Stremlau *et al.*, 2006a). What these studies did not take into the account was the possibility of the existence of more than one TRIM5α-mediated block to viral infection. The fact that TRIM5α was shown to lower reverse transcription of the sensitive virus did not exclude the possibility that it might also act at the later, post-RT stages of infection. This was hinted at by the report of Ylinen *et al.*, (2005) showing the ability of the squirrel monkey TRIM5α to block SIV_{mac} infection after reverse transcription. Furthermore, analysis of viral reverse transcriptase products in the study of Yap *et al.*, (2006), exploring HIV-1 restriction mediated by fusion proteins between RBCC domains of TRIM 1, 18 or 19 and CypA, revealed the ability of these fusion proteins to block HIV-1 replication at two distinct stages of its life cycle; either prior to reverse transcription or just before integration, depending upon the TRIM family member. These findings were recently clarified by Wu *et al.*, (2006) reporting that the proteasome inhibitors rescued HIV-1 reverse transcription products from $TRIM5\alpha_{rh}$-mediated restriction, even though viral infection and generation of 1- and 2-LTR reverse transcription products remained impaired (Wu *et al.*, 2006). This study convincingly showed that TRIM5α acts at more than one step of retroviral life cycle. Thus, the disruption of the proteasomal pathway rescues reverse transcription, but not retroviral infectivity because there are additional TRIM5α-mediated blocks at the post RT stage of infection (Anderson *et al.*, 2006; Wu *et al.*, 2006).

Taken together, these data might suggest the following TRIM5α-antiviral mechanism: After entry into the cell, the retrovirus is recognised by TRIM5α through viral capsid-B30.2 domain interactions. Two distinct antiviral activities of TRIM5α might then follow. In one case, TRIM5α restriction mimics the murine restriction factor Fv1, allowing viral reverse transcription to proceed but blocking the access of viral PICs into the nucleus. This might happen through perturbing the composition, or efficient trafficking, of retroviral RTCs towards the nucleus. As discussed in chapter 1.2.3.4, retroviruses utilise the cellular cytoskeleton on their transit to the nucleus. Binding of TRIM5α might modify or mask the viral sites required for interaction with cellular factors associated with the microtubule network. A second activity might involve the TRIM5α E3 ligase activity. TRIM5α could induce degradation, or prevent the formation, of viral cDNA via proteasome function. Poor performance of either activity would still keep the virus restricted. Ultimately, inhibition of retroviral infection by two independent mechanisms ensures an efficient block to retroviral infection. It would also

ensure restriction in a cell type where, due to the different pattern of protein expression, one of the activities is inactive or less active. The experiments mentioned above, where proteasome inhibition of HeLa cells resulted in no increased viral titre, showed that block to RT is not necessary for viral restriction. However, these effects might be cell-type specific, and testing other cell lines would reveal further insight into the mechanism.

1.3.4.4 Factors involved in TRIM5α-mediated restriction

The only cellular factor thus far identified participating in simian TRIM5α-mediated restriction is cyclophilin A (CypA) (Berthoux *et al.*, 2005b; Keckesova *et al.*, 2006; Stremlau *et al.*, 2006b). CypA was discovered because of attempts to find a gene responsible for Fv1 activity. It was identified in the yeast two-hybrid screen as a binding partner of HIV-1 capsid protein (Luban *et al.*, 1993). CypA belongs to a highly conserved, abundantly expressed cyclophilin family, members of which catalyse the *cis/trans* isomerisation of prolyl peptide bonds (reviewed in Min *et al.*, 2005; Barik, 2006). CypA binds and isomerises the G89-P90 motif within the capsid protein of HIV-1 (Figure 1.13, Gamble *et al.*, 1996; Gitti et al., 1996; Yoo *et al.*, 1997; Bosco *et al.*, 2002; Bosco and Kern, 2004), and via this interaction is efficiently incorporated into HIV-1 particles (Franke *et al.*, 1994; Thali *et al.*, 1994; Ott *et al.*, 1995). Inhibition of CA-CypA interactions either with an immunosuppressive drug (cyclosporine A, CSA), by mutation of either G89 or P90, or by downregulation of CypA decreased HIV-1 replication in human cells (Franke *et al.*, 1994; Thali *et al.*, 1994; Braaten *et al.*, 1996a,b,c; Dorfman and Gottlinger, 1996; Franke and Luban, 1996; Braaten and Luban, 2001; Sokolskaja *et al.*, 2004). Examination of CypA-deficient HIV-1 virions failed to detect any abnormalities in virion components or ultrastructure (Braaten *et al.*, 1996b; Kong *et al.*, 1998; Grattinger *et al.*, 1999; Wiegers *et al.*, 1999) thus casting doubt on the role of incorporated CypA in HIV-1 infectivity. Subsequent studies indicated that although CypA can bind to viral CA in the producer cell during viral assembly, it is the CypA in the target cells that is important for HIV-1 infectivity (Kootstra *et al.*, 2003; Towers *et al.*, 2003; Sokolskaja *et al.*, 2004; Hatziioannou *et al.*, 2005).

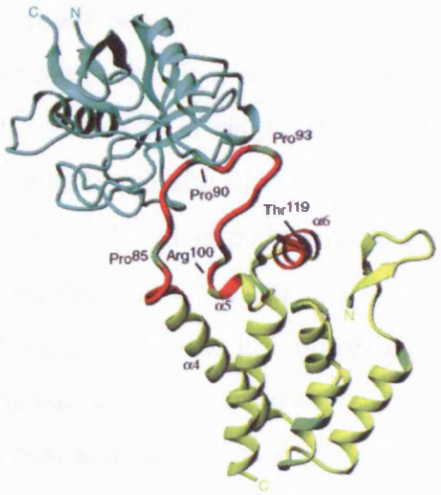


Figure 1.13. Cyclophilin A bound to N-terminal domain of HIV-1 capsid protein. Identification of residues within HIV-1 N-terminal (1-146) CA domain (CA^N) important for binding and catalysis by CypA. CypA (blue) is shown bound to the flexible surface-exposed loop between Pro-85 and Pro-93 of CA^N (red). The rest of CA^N is shown in yellow (Bosco *et al.*, 2002).

Initially it was assumed that HIV-1 capsids required CypA to properly uncoat or efficiently reverse transcribe (Braaten *et al.*, 1996a,b, 2001; Luban, 1996; Bosco *et al.*, 2002; Howard *et al.*, 2003; Bosco and Kern, 2004). However three lines of evidence suggested the possibility of the involvement of a restriction factor in the effect of cyclophilin A: 1) Two mutations in the cyclophilin binding loop of HIV-1, A92E and G94D, alter the sensitivity of HIV-1 replication to cyclosporine in a cell-type specific way. These mutants were originally derived by serial passage of HIV-1 in $CD4^+$ HeLa cells in the presence of a cyclosporine analogue (Braaten *et al.*, 1996a). In these cells, the mutants become cyclosporine-dependent; they do not replicate in the absence of drug, while in the Jurkat human T-cell line these mutations confer cyclosporine-resistance but not drug-dependence. As cyclophilin A is one hundred percent conserved between these cell lines, this differential response to CypA might be explained by the presence of a A92E-, or G94D-specific antiviral protein within HeLa cells. 2) Substitution of the cyclophilin A-binding region in the HIV-1 capsid with that of the macrophage tropic primary isolate HIV-1 Ba-L resulted in a virus that was resistant to the simian inhibitory factor, and efficiently transduced simian cells. The alteration of the CypA region did not affect CypA incorporation into the virions (Kootstra *et al.*, 2003). 3) Cells from rabbit, Rhesus macaque and squirrel monkey showed different permissivities to the very closely related primate lentiviruses HIV-2 and SIV_{mac}. Both of these viruses are products of zoonosis from sooty mangabeys and both were shown not to bind cyclophilin A. Comparing the amino-acid sequence of their capsid protein

revealed differences in the small part of the protein homologous to the cyclophilin A-binding loop of HIV-1 (Ylinen *et al.*, 2005). All of this data suggested that the cyclophilin A-binding loop within HIV-1 might also function as a viral determinant of species-specificity; i.e. it might be the site of the interaction with a potential restriction factor. This observation suggested that experiments examining the effect of CSA on species permissivity to viral infection in various cell lines might be informative. It was found that HIV-1 treated with CSA, but not untreated virus, was able to abrogate restriction of N-MLV in human cells. A model was proposed in which CypA protects HIV-1 capsid from an antiviral activity in human cells (Towers *et al.*, 2003). The effect of CypA on HIV-1 replication was shown to be species-specific. Although treatment of cells with CSA has been shown to decrease the titre of HIV-1 in human cells, it increased the titre of HIV-1 in simian cells implying that while CypA protects HIV-1 from antiviral factor/s in human cells it facilitates restriction in monkey cells (Towers *et al.*, 2003). The observation made in one of the monkey species, specifically owl monkeys, has recently been explained by the identification of the Owl monkey restriction factor as a fusion protein between restriction factor TRIM5α and cyclophilin A (TRIMCyp, Sayah *et al.*, 2004b; Nisole *et al.*, 2004; see chapter 1.3.4.6). It raised the possibility that free CypA is also somehow relevant to TRIM5α restriction of HIV-1 in other primate species where TRIM5α is not fused to CypA. This was confirmed by findings that Rhesus macaque, and African green monkey, cells required CypA for maximal TRIM5α-mediated restriction (Berthoux *et al.*, 2005b; Keckesova *et al.*, 2006; Stremlau *et al.*, 2006b). Depletion of TRIM5α from these cells abolished the effects of CypA on HIV-1 infectivity. In these species, CypA activity has no effect on the ability of TRIM5α to restrict N-MLV or SIV, suggesting that CypA acts on HIV-1 CA rather than on TRIM5α (Berthoux *et al.*, 2005b; Keckesova *et al.*, 2006; Stremlau *et al.*, 2006b). However, in the case of human cells, the stimulatory effect of CypA on HIV-1 replication was shown to be independent of TRIM5α (Keckesova *et al.*, 2006; Sokolskaja *et al.*, 2006a; Stremlau *et al.*, 2006b).

Apart from cyclosporine, another drug, arsenic trioxide, was shown to have an effect on TRIM5α-mediated restriction (Keckesova *et al.*, 2004; Sebastian *et al.*, 2006). Arsenic trioxide (As_2O_3) is known as an effective therapeutic agent for treatment of acute promyelocytic leukemia (Shen *et al.*, 1997). This type of cancer is caused by a chromosomal translocation resulting in the fusion of the promyelocytic leukemia (PML) and retinoic acid receptor (RARα) proteins (de The *et al.*, 1991). PML is a tripartite motif protein (also called TRIM19), related to TRIM5, with roles in apoptosis,

transcription and innate immunity (more about PML in chapter 1.3.4.1; Quignon *et al.*, 1998; Wang *et al.*, 1998a,b; Choi *et al.*, 2006; reviewed in Mann and Miller, 2004; Takahashi *et al.*, 2004). Arsenic trioxide causes the deactivation and/or degradation of PML and PML-RARα, thus eliminating the carcinogenic effect of this fusion protein (Lallemand-Breitenbach, 2001). It was also shown to influence retroviral infection; to stimulate HIV-1 replication (Turelli *et al.*, 2001; Berthoux *et al.*, 2003) and to specifically rescue infectivity of restricted N-MLV and EIAV virus without affecting the unrestricted B- or NB-MLV virus in human cells (Berthoux *et al.*, 2003; Keckesova *et al.*, 2004). The interesting observation of the ability of As_2O_3 to enhance retroviral infection in human cells was recently explained by its effect on TRIM5α. When TRIM5α expression is downregulated in human cells, As_2O_3 is no longer able to influence retroviral restriction in these cells (Sebastian *et al.*, 2006). Overexpression of TRIM5α in otherwise nonrestrictive feline cells, where viral titres are not responsive to arsenic treatment, renders viruses on these cells sensitive to this drug (Keckesova *et al.*, 2004; Sebastian *et al.*, 2006).

TRIM5α orthologs from different species (Berthoux *et al.*, 2005a; Nakayama *et al.*, 2006), mutated TRIM5α (Mische *et al.*, 2005; Perez-Caballero *et al.*, 2005a) and TRIM5 isoforms gamma (Stremlau *et al.*, 2004) and delta (Passerini *et al.*, 2006) have been shown to have a dominant negative activity on TRIM5α-mediated restriction when co-expressed with wild type TRIM5α. In general it can be said that almost any functionally defective TRIM5 protein retaining a coiled-coil domain and capable of binding wild-type TRIM5α might exhibit a dominant-negative effect on this protein. Whether the dominant-negative activity of the short splice variants of TRIM5 is important for modulating TRIM5α activity in *vivo* is unclear at present.

1.3.4.5 Involvement of cytoplasmic bodies in TRIM5α activity

Exogenously-expressed TRIM5α has been shown to be localised in punctuate cytoplasmic structures referred to as "cytoplasmic bodies" (Reymond *et al.*, 2001). Whether endogenous TRIM5α forms these cytoplasmic bodies is unclear, but it is clear that localisation of TRIM5α in these bodies is not required for antiviral activity (Perez-Caballero *et al.*, 2005a; Song *et al.*, 2005a). Stable cell lines expressing TRIM5α sometimes do not form discernible cytoplasmic bodies, yet these cells restrict infection effectively. Moreover, cells treated with geldanamycin, an inhibitor of the heat shock protein Hsp90, which was shown to reduce or eliminate the association of TRIM5α with

cytoplasmic bodies, had no effects on the ability of TRIM5α to restrict retroviral infection (Perez-Caballero *et al.*, 2005a; Song *et al.*, 2005a). Cytoplasmic bodies are likely to be formed in response to high expression levels. Induction of protein expression using sodium butyrate promotes TRIM5α body formation but does not impact on restriction (Perez-Caballero *et al.*, 2005a).

Misfolded or over-expressed proteins are, in the presence of proteasome inhibitors, usually accumulated in cytosolic aggregates which might be later transported via microtubules to the microtubule-organizing centre and concentrated within perinuclear structure known as aggresomes (Johnston *et al.*, 1998, 2002). Aggresomes are often enveloped in vimentin structures and have been shown to contain heat shock proteins, ubiquitin and proteasomal components (Garcia-Mata *et al.*, 2002). Examination of subcellular localisation of inducible nitric oxide synthetase demonstrated, that aggresomes function not only as a site where misfolded or aggregated proteins are directed, but also where functional physiologically-folded proteins are placed in order to regulate their intracellular function (Kolodziejska *et al.*, 2005).

A study by Diaz-Griffero et al., (2006a) showed that human and Rhesus TRIM5α proteins undergo rapid turnover with half-lives of approximately one hour. Their steady-state levels in cell are therefore maintained by continuous synthesis and rapid degradation and it was suggested that cytoplasmic bodies might result when levels of synthesis are greater than the cell's ability to refold or degrade the TRIM5 proteins. Thus cytoplasmic bodies might simply represent aggresomal precursors, linked to the aggresome by microtubular transport (Diaz-Griffero *et al.*, 2006a).

1.3.4.6 Characteristics of TRIMCyp

The mechanism of action of TRIMCyp is suggested by its structure. The B30.2 domain, which governs restriction specificity by serving as a capsid recognition domain in the TRIM5α molecule, is almost precisely replaced by CypA in the TRIMCyp chimera. This results in the exchange of one virus-binding domain for another. Stable, polymeric viral capsids with properly folded amino-terminal CA subunits are essential for recognition by TRIMCyp (Forshey *et al.*, 2005). Kinetic studies revealed that inhibition of incoming HIV-1 capsids by TRIMCyp occurs within 15 minutes of their delivery to the target cell. This suggests that the sub-viral structure that is targeted either exists only transiently after virus entry, or moves quickly to a location where restriction cannot occur (Perez-Caballero *et al.*, 2005b). As is the case for TRIM5α, TRIMCyp has a rapid

turnover of about one hour, and this is dependent on the proteasome and the presence of a RING domain (Diaz-Griffero *et al.*, 2006a). Its activity does not require cytoplasmic bodies (Perez-Caballero *et al.*, 2005b). Capsid binding occurs most efficiently when TRIMCyp is trimeric, and has intact coiled-coil and cyclophilin A domains. Its antiviral function also depends upon the B-box 2 domain (Diaz-Griffero *et al.*, 2006b).

The *CypA* insertion appears to be unique to owl monkeys. It arose after the divergence of the New and Old World primates when a LINE-1 retrotransposon catalysed the insertion of a *CypA* cDNA into the TRIM5 locus. It serves as the first vertebrate example of a chimeric gene mediated by this mechanism of gene shuffling (Nisole *et al.*, 2004; Sayah *et al.*, 2004b; Ribeiro *et al.*, 2005).

1.3.4.7 TRIM5α polymorphism and gene therapy

Human TRIM5α exerts only modest restriction of HIV-1, and is unable to block productive infection in human cells (Hatziioannou *et al.*, 2003; Stremlau *et al.*, 2004). However, it was hypothesised that polymorphism within the TRIM5 gene might explain the observed variability of human susceptibility to HIV-1 infection and disease progression, and might result in increased restriction of HIV-1 infection in some individuals. This notion led to the investigation of polymorphism in the TRIM5 gene from primary lymphocytes of many HIV-1 infected, or exposed, seronegative subjects (Speelmon *et al.*, 2006). These and similar studies observed no significant effect of individual TRIM5α nonsynonymous mutations on the HIV-1 susceptibility of human $CD4^+$ T-cells (Goldschmidt *et al.*, 2006; Javanbakht *et al.*, 2006a; Speelmon *et al.*, 2006).

Studies of intra-species variation within TRIM5α coding sequences of two distinct Old World monkey species, Rhesus macaques and sooty mangabeys, revealed very extensive polymorphism within these species resulting in the presence of multiple TRIM5α alleles (Newman *et al.*, 2006). Interestingly, certain alleles were more closely related to orthologs of other species than to one another. This led to the conclusion that the TRIM5 locus of Old World primates was shaped by long-term balancing selection, which is rare and can be seen, for example, in the case of the evolution of the MHC locus and ABO blood group system (Newman *et al.*, 2006). Since diversity is necessary, in terms of antiviral protection, the existence of multiple TRIM5α alleles might have given these species the advantage of recognising more viral epitopes, thus enabling them to fight off infections by a broader array of viruses.

The ability of Rhesus macaque TRIM5α to strongly restrict HIV-1 replication *in vitro* highlighted its potential as a protective molecule for gene therapy applications. This led to initiation of experiments that showed that TRIM5α_{rh}-transduced human $CD34^+$ hematopoietic progenitor cells produce transgenic macrophages which are highly resistant to HIV-1 infection and do not present any apparent phenotypical or functional defects (Anderson and Akkina, 2005). Such cells could theoretically be used to repopulate the immune system of an HIV-1 infected individual.

1.3.5 APOBEC3G

Another of the antiviral mechanisms was discovered from studies of the HIV-1 viral infectivity factor (Vif) protein. The *vif* gene was shown to be dispensable for HIV-1 replication in certain cell lines (termed "permissive"), but was very important for replication in other cells (termed "nonpermissive"). Fusion of these cell lines showed that the phenotype is dominant (Madani and Kabat, 1998; Simon *et al.*, 1998). The gene responsible for this antiviral activity was identified by subtractive cloning as apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) (Sheehy *et al.*, 2002). This protein belongs to the APOBEC family of cytidine deaminases, which target cytosines in RNA and/or DNA and convert them to uracils. A well characterised member of this group is AID (activation-induced deaminase), which regulates somatic hypermutation and isotype class switching in B-cells, and APOBEC1 that regulates the expression of different isoforms of Apolipoprotein B in the small intestine. APOBEC3G is incorporated into HIV-1 particles (Kao *et al.*, 2003; Zennou *et al.*, 2004) in the producer cell through the interaction with the nucleocapsid portion of Gag polyprotein (Alce and Popik, 2004; Cen *et al.*, 2004; Luo *et al.*, 2004; Schafer *et al.*, 2004) and acts during HIV-1 reverse transcription in the target cell. It was found to act on a single-stranded DNA intermediate and it mutates as many as 4% of the C residues of the minus-strand viral DNA of vif-minus HIV-1 (Harris *et al.*, 2003; Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Yu *et al.*, 2004). The C to U mutations in the minus strand produce G to A mutations in the complementary cDNA strand. Viral genomes bearing these mutations are either degraded by cellular DNA-repair enzymes or are unable to direct the synthesis of functional viral proteins due to the introduction of premature stop codons and heavy mutations. Other APOBEC family members were also found to inhibit HIV-1 infection, such as APOBEC3F, 3B and 3DE (Bishop *et al.*, 2004; Liddament *et al.*, 2004; Zheng *et al.*, 2004; Dang *et al.*, 2006; reviewed in Goff,

2004; Zheng *et al.*, 2005; Malim, 2006; Perez and Hope, 2006). However, it is becoming increasingly clear from recent data that a strong antiviral effect of APOBEC proteins can also be achieved in the absence of detectable hypermutation. Mutated APOBEC proteins that are unable to function as cytidine deaminases still retain substantial levels of antiviral activity, correlating with disruptions in viral cDNA accumulation (Newman *et al.*, 2005; Bishop *et al.*, 2006a). From the data available, it is not yet possible to determine whether this effect is due to the degradation of reverse transcripts, or whether these products are simply not made (reviewed in (Holmes *et al.*, 2007).

The HIV protein Vif impairs the ability of APOBEC3G to enter virions (Mariani *et al.*, 2003). Vif was shown to bind directly to APOBEC3G and induce its proteosomal degradation through linking APOBEC3G with a ubiquitin-ligase complex that contain several cellular proteins such as elongin B, elongin C, cullin-5 (CUL5) and ring-box-1 (RBX1, Conticello *et al.*, 2003; Marin *et al.*, 2003; Sheehy *et al.*, 2003; Stopak *et al.*, 2003; Yu *et al.*, 2003). HIV-1 Vif is able to eliminate APOBEC3G in humans and chimpanzees but not those of Old World monkeys like African green monkeys (Bishop *et al.*, 2004). A single amino-acid of human APOBEC3G (aspartic acid at position 128) was shown to be responsible for its sensitivity to inactivation by HIV-1 Vif (Bogerd *et al.*, 2004; Mangeat *et al.*, 2004; Schrofelbauer *et al.*, 2004).

Several members of the APOBEC family were shown to be active not only against retroviruses but also against hepatitis B virus (Turelli *et al.*, 2004) and endogenous retroelements (Esnault *et al.*, 2005) using both editing and non-editing mechanisms (reviewed in Holmes *et al.*, 2007).

1.3.6 ZAP

The zinc finger antiviral protein (ZAP) was identified by a screen of a rat cDNA library as a gene conferring resistance to the infection of cells by MLV (Gao *et al.*, 2002). ZAP is an RNA-binding protein that contains a cluster of four CCCH-type zinc fingers. An analysis to determine the step at which ZAP blocked virus infection revealed that while it has no effect on MLV entry, DNA synthesis or integration, there is a dramatic and specific loss of appearance of viral RNAs in the cytoplasm (Gao *et al.*, 2002). Mutational studies suggested that the zinc fingers are involved in direct binding of ZAP to MLV mRNA (Guo *et al.*, 2004). ZAP was found to interact with exosome; a cytoplasmic complex of about ten proteins responsible for mRNA turnover. It directly

binds exosome's hRrp46p component and targets the viral RNA for exosome-mediated destruction (Guo *et al.*, 2007). In addition to its inhibition of MLV, ZAP was shown to potently inhibit the replication of several members of *Alphaviruses* (Bick *et al.*, 2003).

1.4 Project introduction

The aim of my PhD research was to identify host factors impacting on viral permissivity, and to understand the molecular mechanisms involved. Identification of TRIM5α as a factor responsible for HIV-1 restriction in Rhesus macaque cells allowed us to test if TRIM5α is also responsible for Ref1 activity in human cells and Lv1 activity in monkey cells, and thus if Lv1 and Ref1 are species-specific variants of a single restriction factor, or if these activities are encoded by separate restriction systems. Furthermore, we have examined whether the properties of TRIM5α cloned from different species account for the characteristic restriction patterns seen in corresponding cell lines. We also wanted to examine the contribution of shorter isoforms, specifically TRIM5δ, to retrovirus restriction. We sought to identify other host factors involved in the TRIM5α restriction pathway. One host factor known to directly bind retroviral capsid is cyclophilin A. Since mutations at the base of the CypA loop (H87Q), or in the CypA binding site modulate the susceptibility to different TRIM5α variants, we decided to analyse the role of CypA in TRIM5α-mediated restriction. Given the fact that arsenic trioxide was shown to stimulate retroviral infectivity, we also wanted to examine if this stimulatory effect involves suppression of TRIM5-mediated restriction activity and to uncover details of the involvement of arsenic in TRIM5α-mediated restriction.

The results presented in this work clearly show that TRIM5α is an important component of innate antiviral defense mechanisms. Proper understanding of its function and the way it interacts with other cellular factors could reveal ways to enhance, induce, improve or broaden the activities of this aspect of antiviral innate immunity to our advantage.

Chapter 2

Materials and Methods

2.1. Cell Culture

2.1.1 Cell lines

Cell line	Description
293T	Human embryonic kidney cell line expressing the Simian Virus 40 (SV40) Large T antigen.
CRFK	Crandall-Reese feline kidney cell-line established from *Felis catus* species
CRFKhuT5α	CRFK cells stably expressing human TRIM5α
CRFKHAhuT5α	CRFK cells stably expressing human TRIM5α fused to an HA-tag
CRFKAgmT5α	CRFK cells stably expressing African green monkey TRIM5α
CRFKHAAgmT5α	CRFK cells stably expressing African green monkey TRIM5α fused to an HA-tag
CRFKhuK10R	CRFK cells stably expressing human TRIM5α mutant K10R
CRFKhuKK263,264RR	CRFK cells stably expressing human TRIM5α mutant KK263,264RR
CRFKTRIMCyp	CRFK cells stably expressing Owl monkey TRIMCyp
CV1	African green monkey kidney cell-line *(Cercopithecus aethiops tantalus)*
CV1dT5	CV1 cells stably knocked-down for Agm TRIM5α expression
CV1dcypA	CV1 cells stably knocked-down for cyclophilin A expression
FRhK	Fetal Rhesus macaque monkey kidney cell-line
FRhKdT5	FRhK cells stably knocked-down for Rhesus macaque TRIM5α expression
FRhKdcypA	FRhK cells stably knocked-down for cyclophilin A expression
HeLa	Human epithelial cells established from a cervical carcinoma transformed by human papillomavirus 18
TE671	Human rhabdomyosarcoma cell-line
TEdT5	TE671 cells stably knocked-down for human TRIM5α expression
TET5δ	TE671 cells stably over-expressing the human TRIM5δ isoform

Table 2.1. Cell lines.

All cells are adherent. CFRK cells are permissive for all of the viruses tested including N-MLV, B-MLV, SIV_{mac}, HIV-2 and HIV-1. HeLa, TE671 and 293T cells were obtained from the American Type Culture Collection (ATCC). CRFK were donated by Yasuhiro Ikeda, University College London. FRhK cells were obtained from the Centro Substrati Cellulari, Brescia, Italy. CV1 cells were a kind gift of P. Jat, Ludwig Institute for Cancer Research, London.

2.1.2 Thawing cells

Cells were removed from liquid nitrogen or –80°C freezer and thawed rapidly at 37°C. Thawed cells were then added to 20 ml of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, U.K.) with 10% fetal calf serum (FCS, Biosera, U.K.). Next day media was changed for 10 ml of fresh DMEM media with 10% FCS.

2.1.3 Passaging cells

All cell lines, except 293T, were maintained in DMEM (Invitrogen, U.K.) with 10% FCS (Biosera, U.K.) in 5% CO_2 at 37°C. 293T cells were maintained in DMEM (Invitrogen, U.K.) with 15% FCS (Biowest, France) in 10% CO_2 at 37°C. No antibiotics were added. Cells were split 1:5 to 1:20, depending on cell density and rate of growth, two or three times per week.

2.1.4 Freezing cells

Cells from confluent 10 cm plate were trypsinised, centrifuged at 1200 rpm for 5 minutes and resuspended in 3 ml of cold FCS (Biosera, U.K.) containing 10% dimethyl sulphoxide (DMSO, Sigma, U.K.). Cells were aliquoted into cryovials (Nunc, USA) and gradually cooled to –80°C in an isopropanol-containing cryo-container (Nalgene, USA) before being transferred to liquid nitrogen.

2.1.5 Transfection and viral vectors

Vesicular stomatitis virus glycoprotein (VSV-G) – pseudotyped MLV (N, B), EIAV, HIV-2, SIV_{mac}, and HIV-1 GFP-encoding vectors were prepared by transient triple transfection of 293T cells with FuGENE-6 (Roche) according to a protocol by G.J.Towers. The transfection mixture contained 1μg pMDG (encoding the pantropic VSV-G envelope), 1μg of the packaging vector (Gag-Pol expression construct from the virus of interest) and 1.5 μg of the marker-encoding transfer vector in 200 μl Opti-MEM (Invitrogen, U.K.) with 18 μl FuGENE-6. The mixture was incubated at room temperature for 15 minutes before being added dropwise to the confluent 293-T cells in 10 cm petri dish with 8 ml of DMEM medium (Invitrogen, U.K.) and 15% fetal calf serum (Biowest, France).

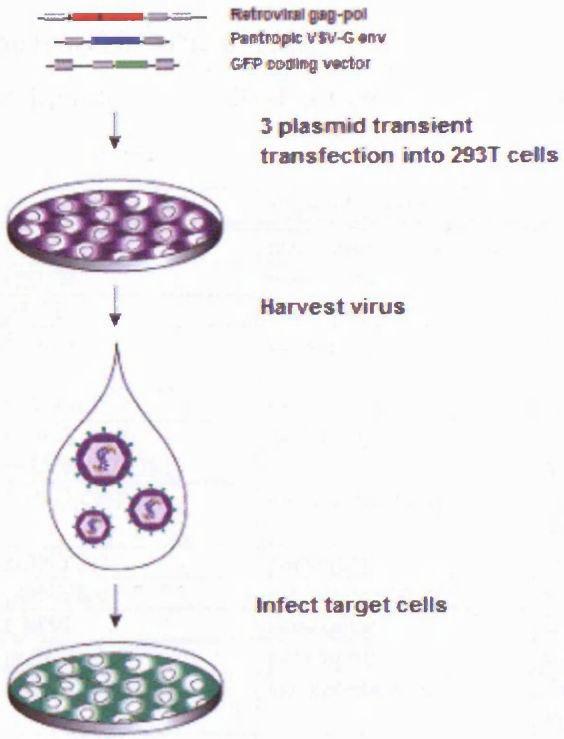


Figure 2.1. Schematic drawing of viral vector production. Modified from http://www.clontech.com/images.

Transfected 293-T cells were incubated overnight at 37°C and with 10% CO_2. Media was changed after 24 hrs, and supernatant was harvested at 48, 72 and 96 hours post-transfection, passed through a 0.45 μm filters (Vivascience, U.K.), aliquoted and stored at – 80°C (Figure 2.1). Marker-encoding transfer vectors encoded green fluorescent protein (GFP) or puromycin resistance gene (puro). The following plasmids were used for the packaging: CMVi Gag-Pol for NB-tropic MoMLV and its derived vectors, N Gag-Pol (pCIG3-N) for N-MLV, B Gag-Pol (pCIG3-B) for B-MLV, pONY3.2 for EIAV, HIV-2 pack (pSVRdNBDM) for HIV-2 (a kind gift of A.M. Lever, see Griffin *et al.*, 2001), MVP5180 for MVP, MVP5180-G89V for MVPG89V, 92BR for 92BR HIV-1, 92BRG89V for 92BR-G89V HIV-1, Bal for HIV-1 Bal, SIV3+ for SIV_{mac}, p8.91 for HIV-1 and p8.91G89V for G89V HIV-1. The following plasmids were used as a marker-encoding vector: pCNCG for MLV GFP expression, CSGW for HIV-1 GFP expression, pONY8.0 for EIAV GFP expression, HIV-2 GFP plasmid for HIV-2 GFP expression. HIV-2 GFP plasmid was derived from pSVRdNBPuro (kindly provided by A.M. Lever, see Griffin *et al.*, 2001) by exchanging the SV40-Puro cassette with a CMV-GFP cassette from CNCG. SIV-GFP was used for making SIV_{mac} GFP, $CT5_{\delta}CR$ for human TRIM5δ (TRIM5δ cDNA was kindly provided by P.G. Pelicci), siT5/CSPW for expression of siRNA against TRIM5 (a kind gift of S.J.Wilson, see Ylinen *et al.*, 2005), R-P/CSPW for expression of siRNA against LacZ (a kind gift of S.J.Wilson), RETROcypA/MLV for expression of siRNA against cyclophilin A, $CT5_hCR$ for human TRIM5α, $CT5_{mac}CR$ for Rhesus macaque TRIM5α (kindly provided by L.M.J.Ylinen), $CT5_{HA\text{-}h}CR$ for human TRIM5α fused to HA-tag, $CT5_{Agm}CR$ for African green monkey (Agm) TRIM5α, $CT5_{HA\text{-}Agm}CR$ for Agm TRIM5α fused to HA-tag, $CT5_{T\text{-}C}CR$ for owl monkey TRIMCyp (gene for TRIMCyp kindly given by J.P.Stoye, see Nisole *et al.*,

2004), $CT5_{ZK8}CR$ for human TRIM5α mutated in KK263,264RR, $CT5_{GT312}CR$ for human TRIM5α mutated in K10R (For plasmid reference see Naldini *et al.*, 1996; Bainbridge *et al.*, 2001; Besnier *et al.*, 2002; Hatziioannou *et al.*, 2003).

Name of plasmid	Type of plasmid	Encoded protein	Vector produced*
CSGW	marker-encoding vector	GFP, Amp^r	GFP or Puro encoding HIV-1
CSPW	marker-encoding	$Puro^r$, Amp^r	
p8.91	packaging construct	Gag, Pol, Tat, Rev, Amp^r	
SIV3+	packaging	Gag, Pol, Tat, Rev, Vif, Vpx, Vpr, Amp^r	GFP encoding SIV_{mac}
SIV-GFP	marker-encoding	GFP, Amp^r	
HIV-2 pack (pSVRdNBDM)	packaging	Gag, Pol, Tat, Rev, Nef, Vif, Vpx, Vpr, Amp^r	GFP encoding HIV-2
HIV-2 GFP	marker-encoding	GFP, Tat, Rev, Nef, Vif, Vpx, Vpr, Amp^r	
pONY3.2	packaging	Gag, Pol, Tat, Rev, Amp^r	GFP encoding EIAV
pONY8.0	marker-encoding	GFP, Amp^r	
CMVi	packaging	Gag, Pol, Amp^r	GFP or Red Express encoding MLV
pCIG3	packaging	Gag, Pol, Amp^r, $Blasti^r$	
CNCR	marker-encoding	Red Express Fluorescent Protein, Neo^r, Amp^r	
CNCG	marker-encoding	GFP, Neo^r, Amp^r	

Table 2.2. Plasmids used for vector production. * In addition to marker-encoding and packaging plasmid also envelope-encoding plasmid (in our study it is pMDG) is needed for vector production.

2.1.6 Drugs

Cyclosporine A (CsA, Sandoz, Frimley, U.K.) was prepared in dimethyl sulfoxide to a 1 mM stock concentration and later diluted in tissue culture medium to the indicated concentrations prior to each experiment. The drug was stored in a dark place at room temperature.

0.1 M As_2O_3 (Sigma) was prepared in 1 N NaOH, diluted to 1mM in PBS, pH adjusted to 7.0 with HCl and filtered through a 0.2 μm filter (Vivascience). The drug was then kept at 4°C for up to 3 months without loss of activity.

Polybrene was dissolved in double distilled water to a 5 mg/ml stock concentration and filtered through 0.2 μm filter (Vivascience). It was later diluted in tissue culture medium to a final concentration of 5 μg/ml.

Puromycin was dissolved in double distilled water to a 2.5 mg/ml stock concentration and filtered through 0.2 μm filter (Vivascience). It was later diluted in tissue culture medium to a final concentration of 1 μg/ml for human cells and 4-8 μg/ml for simian cells.

2.1.7 Infection assays

Target cells were seeded in either 24-well (0.25 x 10^5 cells/well) or 6-well (10^5 cells/well) plates. For virus titration experiments, cells were inoculated with 3-fold serially diluted virus stocks that express a GFP reporter gene (either N-MLV GFP, B-MLV GFP, HIV-1 GFP, HIV-2 GFP, SIV_{mac} GFP or EIAV GFP) in the presence of 5 μg/ml polybrene. Infected target cells were enumerated 48 h later by FACS analysis (Facs Calibur, Becton Dickinson) and Cell Quest software. GFP was recorded on the fluorescence FL1 channel and a total of 10,000 to 50,000 cells per sample were analysed. Murine Leukaemia Virus (MLV) titres were equalized on permissive CRFK cells and, where relevant, titres are described as CRFK infectious units per millilitre. Lentiviral reverse transcriptase activity was measured using an ELISA assay (Cavidi Tech, Uppsala), according to the manufacturer's protocol. Where indicated, cells were treated with As_2O_3 or CSA at the time of infection.

2.1.8 Deriving clonal cell populations

TE671 or CRFK cells stably expressing MLV vectors were generated by infecting CRFK, or TE671 cells at high MOI with a Moloney NB-MLV based vector encoding red fluorescent protein (RFP) and either human TRIM5α, human TRIM5α mutants, Agm TRIM5α or human TRIM5δ. 48 hours later, cells were trypsinised and cell density was determined using a neubauer-improved haemocytometer. Cultures were diluted to 2 cells/ml and cultured in 24-well plates containing 500 μl/well. Cells were left for two weeks to expand and single clones were screened by fluorescent microscopy and analysed by flow cytometry for a single RFP fluorescent population as an indication of clonality. They were then tested for their ability to restrict virus by titration of viral stocks (section 2.1.7).

TE671 or CRFK cells stably expressing short hairpin RNA (shRNA), for TRIM5 or cyclophilin A downregulation, were generated by infecting TE671, CV1 or FRhK cells at high MOI with a retroviral HIV-1-based CSPW vector (siT5/CSPW) containing puromycin resistance gene and encoding shRNA against TRIM5 or with a retroviral MLV-based pSUPERretro vector (RETROcypA/MLV) containing a puromycin resistance gene and encoding shRNA against cyclophilin A. 48 hours post-infection puromycin dihydrochloride from *S. alboniger* (Sigma) was added to cultures at a final concentration of 1 μg/ml on human cells (TE671) or 4-8 μg/ml on simian cells (FRhK

and CV1). Cell were left in the presence of puromycin for one or two weeks (media was changed and supplied with fresh puromycin once in three days) until the sufficiently expanded, puromycin-resistant multi clonal population was derived. In the case of deriving single cell clones, 96 hours after the addition of puromycin dihydrochloride single clonal cell populations were derived by dilution as described above in the continued presence of puromycin. Single or multi clonal populations were then tested either for functional restriction of different MLVs or for the sufficient decrease of cyclophilin A protein levels by western blot. The puromycin-selected cells were then cultured in medium supplemented with puromycin.

2.2 Molecular Biology

2.2.1 Oligonucleotides

Oligo Name	Oligo Sequence	Description
GT269	5'CAGAC**GAATT**CCACCATGGCTTCTGGAATCCT GGTTAATG-3'	TRIM5a fwd EcoR1
GT270	5'ATCG**TTCGAA**TCAAGAGCTTGGTGAGCACAGA G-3'	TRIM5a rev Csp45I
GT303	5'ATCG**TTCGAA**TTAGGCGTAGTCGGGCACGTCG TAGGGGTAAGAGCTTGGTGAGCACAGAGTCA-3'	TRIM5a-HA rev Csp45I
GT296	5'CAGAC**GAATT**CCACCATGGCTTCTGGA ATCCTG-3'	TRIM5 fwd Ecor1 short
GT386	5'TCGAT**TTCGAA**TTAAAGTTGTCCACAGTCAGC AAT-3'	Owl monkey TRIMCyp rev, Csp45I
ZK8sense	5'ACGGAGAACGTGACCTTGAGAAGACCAGAAA CTTTTCCAAAAAAT-3'	change KK263,264RR in huT5α sense
ZK8anti-sense	5'ATTTTTTGGAAAAGTTTCTGGTCTTCTCAAGGT CACGTTCTCCGT-3'	change KK263, 264RR in huT5α antisense
GT312sense	5'ATCCTGGTTAATGTAAGGGAGGAGGTGACCTG C-3'	change K10R in huT5α sense
GT312anti-sense	5'GCAGGTCACCTCCTCCCTTACATTAACCAGGAT -3'	change K10R in huT5α antisense
GT337	5'GATCCCCGGGTTCCTGCTTTCACAGATTCAAGA GATCTGTGAAAGCAGGAACCCTTTTTGGAAA-3'	anti CypA hairpin for pSuper, sense
GT338	5'AGCTTTTCCAAAAAGGGTTCCTGCTTTCACAGA TCTCTTGAATCTGTGAAAGCAGGAACCCGGG -3'	anti CypA hairpin for pSuper, antisense
GT308	5'TCGAT**TTCGAA**CTACTTGGGAGGCTGAGGCAG GAG-3'	TRIM5δ rev Csp45I

Table 2.3. Oligonucleotides. Restriction sites are in bold. Kozak sequences are underlined.

2.2.2 Molecular cloning

- $CT5_hCR$: The human TRIM5α gene was amplified by PCR from the human IMAGE clone no. 6160154 (MRC Gene Service, Cambridge, U.K.) using forward primer GT269 and reverse primer GT270. It was cloned into the MLV vector CFCR between BclI and Csp45I sites such that it was expressed under the control of the Moloney MLV LTR in infected cells (see Figure 2.2). The CFCR vector was first cut with BclI, blunted by T4-DNA polymerase according to manufacturer's protocol (Promega, U.K.) and subsequently cut with Csp45I, thus cutting out the gene for Fv1. The PCR fragment encoding human TRIM5α was cut with Csp45I, the Csp45I site was introduced into the PCR product by the reverse primer, and ligated into the CXCR backbone. The CXCR vector also encodes red fluorescent protein (RFP, Clontech) under the control of a CMV promoter.

- $CT5_{Agm}CR$: Agm TRIM5α was PCR amplified from a cDNA library derived from Agm *Cercopithecus aethiops tantalus* sp. CV1 cells by using primers GT296 and GT270 and cloned into CXCR as above.

- The CXCRdEcoR1 plasmid was derived from the CFCR plasmid in order to make cloning into this vector easier. The CFCR plasmid encodes two restriction sites for EcoR1 (see Figure 2.2). In CXCRdEcoR1 we deleted the EcoR1 site at position 5736 of the CFCR vector. To do this we cut out the part of the vector containing the EcoR1 site at 1557 with SnaB1 and religated the vector (see Figure 2.2). Vector was then cut with EcoR1, blunted with T4-DNA polymerase (Promega, U.K.) and religated. The vector was then reconstituted by cloning back the SnaB1 fragment. We then cut out the Fv1 gene encoded by this vector using the 5' EcoR1 site and the 3' Csp45I site. These two sites were used for subsequent insertion of genes of interest.

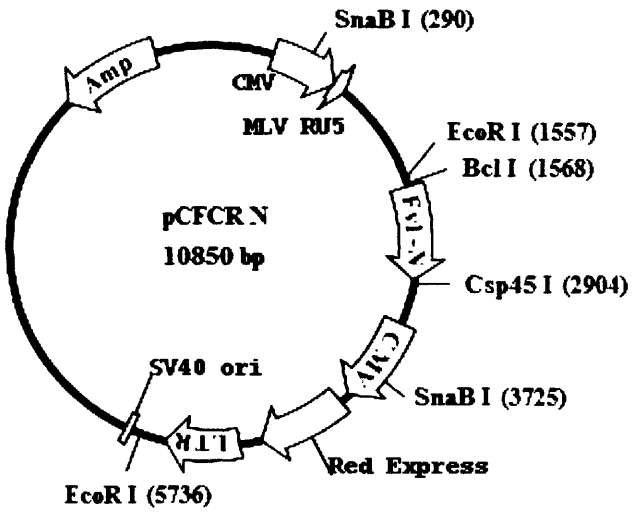


Figure 2.2. Circular map of CFCR plasmid. CFCR is an MLV vector derived from the CNCG vector made by Oxford Biomedica, U.K. CMV drives expression of the RNA in the packaging cells. The MLV LTR is reconstituted during reverse transcription and drives expression of Fv1-N in target cells. The CXCRdEcoR1 plasmid used in this thesis was derived from CFCR by deleting the EcoR1 site at position 5736 and by replacing the Fv1 gene with our genes of interest.

- $CT5_{\delta}CR$: Human TRIM5δ gene was amplified by PCR from a plasmid kindly provided by P.G. Pelicci using primers GT269 and GT308 and cloned into CXCRdEcoR1 using EcoR1 and Csp45I sites within primers.

- $CT5_{HA\text{-}h}CR$ and $CT5_{HA\text{-}Agm}CR$ vectors expressing human TRIM5α and Agm TRIM5α fused to haemagglutinin tags at their C-terminus were generated by PCR from

$CT5_hCR$ and $CT5_{Agm}CR$, respectively, using primers GT269 and GT303 in the case of $CT5_{HA\text{-}h}CR$ and GT296 and GT303 in the case of $CT5_{HA\text{-}Agm}CR$ and cloned into CXCRdEcoR1 using the EcoR1 and Csp45I sites as above.

- $CT5_{T\text{-}C}CR$: The Owl monkey TRIMCyp cDNA was kindly provided by J.P.Stoye, see Nisole *et al.*, 2004. The cDNA for TRIMCyp was PCR amplified using primers GT296 and GT386, cut with EcoR1 and Csp45I and re-cloned into the CXCRdEcoR1 vector cut with the same restriction enzymes.

- $CT5_{GT312}CR$ and $CT5_{ZK8}CR$: Human TRIM5α mutants K10R and KK263,264RR were made by site-directed mutagenesis of wild type human TRIM5α encoded within the pBluSKP (pBS) vector. pBS was cut with Not1, blunted with T4-DNA polymerase (Promega) according to the manufacturer's protocol and then cut with EcoR1. The PCR fragment of wild type human TRIM5α was cut with EcoR1 (the EcoR1 site was introduced into the PCR product with the forward primer) and ligated into the pBS backbone. The TRIM5α K10R mutant was made using primers GT312 sense and antisense and mutant KK263,264RR was made using primers ZK8 sense and antisense by site-directed mutagenesis (section 2.2.6). The resulting mutant TRIM5α genes were cut out of pBS with EcoR1 and Csp45I restriction enzymes and cloned into CXCRdEcoR1 opened with the same enzymes.

- RETROcypA/MLV: Oligonucleotides GT337 and GT338 encoding shRNA targeting a sequence within CypA (GGGTTCCTGCTTTCACAGA) were ligated into the pSUPERretro MLV expression vector using BglII and HinDIII restriction sites (as described by Sayah *et al.*, 2004b).

All constructs were verified by DNA sequencing (Lark Technologies, U.K.). The sequence of human, Agm and Rhesus macaque TRIM5α was determined by sequencing three independent clones. DNA analysis was performed with DNA Dynamo (Blue Tractor Software, U.K.).

2.2.3 Disruption of TRIM5α expression

- Transient disruption of gene expression: Synthetic short interfering RNA (siRNA) oligonucleotide duplexes (Qiagen, U.K.) were targeted to sequences within human TRIM5 (AAGCTCAGGGAGGTCAAGTTG) as described by Stremlau *et al.*, 2004. HeLa cells were transfected with 60 pmol (20 pmol/μl) of the specific RNA duplex using Oligofectamine (Invitrogen) according to the manufacturer's instructions. 48 hours after siRNA transfection the cells were infected with GFP-encoding MLV (N, B), HIV-1, HIV-2, SIV_{mac} or EIAV-based vectors. Green fluorescing, infected cells were enumerated by FACS 48 hours after infection. Control siRNA transfections were performed with a negative control siRNA against the μ2 subunit of AP2 (a kind gift of Alberto Fraile-Ramos; Fraile-Ramos *et al.*, 2003).

- Stable disruption of gene expression: Cells were infected at high multiplicities with HIV-1 based CSPW vector (siT5/CSPW) encoding shRNA targeted against human or Agm TRIM5alpha (AAGCTCAGGGAGGTCAAGTTG, a kind gift of S.J.Wilson) or with MLV based pSUPERretro vector (RETROcypA/MLV) encoding shRNA against cyclophilin A (GGGTTCCTGCTTTCACAGA). This vector was packaged into VSV-G pseudotyped HIV-1 or MLV virus by transfection of 293T cells as described above and a stable knock-down of TRIM5 or cyclophilin A was generated in TE671, CV1 and FRhK cells as described above. Efficient and specific knock-down was tested either by western blot analysis (in the case of cyclophilin A) or genetically, by infection with different MLVs (in the case of TRIM5).

2.2.4 Introduction of plasmid DNA into *E. coli*

- Electroporation: 1-100 ng of plasmid in a volume not exceeding 2 μl was mixed with 80 μl of cold electrocompetent XL-1 Blue *Escherichia coli* (kindly provided by L.M.J.Ylinen) and incubated on ice for 1 minute. The mixture was then transferred to an electroporation cuvette (Biorad, U.S.A.) and electroporated in a Gene Pulser™ (Biorad, U.S.A.) set at 25 μF, 2,5 kV and 200 Ω. After the pulse, 1 ml of SOC media (Invitrogen, U.K.) was added to the mixture. The whole reaction was then transferred to a falcon tube and shaken at 37 °C for 1 hour. Finally, the cells were plated on LB-agar plates containing 50 μg/ml ampicillin or kanamycin.

- Heat-shock: 1-200 ng of plasmid in a volume not exceeding 10 µl was mixed with 100 µl of heat-shock competent TOP10, HB101 or DH5α *Escherichia coli* strains (Invitrogen, U.K. or kindly provided by L.M.J.Ylinen) and incubated on ice for 15 minutes. Cells were then heat shocked for 30 seconds at 42 °C and cooled on ice for 2 minutes. The mixture was then either directly streaked on to LB-agar plates containing 50 µg/ml ampicillin or 200 µl of SOC media (Invitrogen, U.K.) was added, the mixture was shaken at 37 °C for 45 minutes and then transferred to LB-agar plates containing 50 µg/ml kanamycin or ampicillin.

2.2.5 Plasmid DNA mini-preps

Bacterial clones were streaked on LB-agar plates containing relevant antibiotics and left overnight at 37 °C. The next day, they were scraped off using sterile loops (Greiner Bio-one, Germany) and mini-preps of plasmid DNA were produced using the Miniprep Kit (Qiagen, U.K.) according to the manufacturer's instructions.

2.2.6 PCR-amplification and ligation

DYAD or TETRAD PCR-machines (MJ Research, U.S.A) were used for basic PCR techniques and PCR mutagenesis using Pfx Platinum DNA-polymerase (Invitrogen, U.K.), generating blunt-end PCR products, according to the manufacturer's instructions. The PCR reaction mixture had a total volume of 50 µl and consisted of:

5 µl of DNA-polymerase buffer (10x)
1 µl of plasmid DNA (app. 100ng)
5 µl of forward primer (10 µM)
5 µl of reverse primer (10 µM)
2 µl of supplied Mg_2SO_4
2 µl of dNTP (10mM)
0.5 µl of Pfx Platinum polymerase
29.5 µl of sterile water

• The basic PCR: 50ng of plasmid DNA or 100ng of genomic DNA was used to PCR-amplify the target region, of approximately 1500 nucleotides, by using the following PCR cycle:

1. 3 minutes at 94 °C
2. 30 seconds at 94 °C
3. 1 min at 60 °C
4. 2 min at 68 °C
5. Steps 2-4 an additional 30 times
6. 10 minutes at 70 °C

The PCR product was resolved by agarose gel electrophoresis, purified from the gel using a QIAquick gel extraction kit (Qiagen, U.K.), cut with relevant restriction enzymes and ligated into a plasmid backbone using T4-DNA ligase (Promega) as recommended by the manufacturer's protocol. Alternatively, PCR product was directly cloned into a TOPO expression plasmid using the Zero Blunt TOPO Kit (Invitrogen, U.K.) according to the manufacturer's protocol.

• PCR mutagenesis: 100-200 ng of plasmid DNA was used for site-directed mutagenesis, using the following PCR cycle:

1. 3 minutes at 94 °C
2. 30 seconds at 94 °C
3. 30 seconds at 60 °C
4. 12 min at 68 °C
5. Steps 2-4 an additional 20 times
6. 20 minutes at 70 °C

The extension time of 12 minutes is applicable for plasmids smaller than 6000 nucleotides. In some cases the plasmid was about 10 Kb so the extension time was extended to 20 minutes. DpnI enzyme (1 µl) was then added to the reaction, which was incubated for 2 hours at 37 °C. 2 µl of the reaction was then transformed into *Escherichia coli* bacteria using heat-shock.

2.2.7 Quantitative PCR

TaqMan PCR to measure viral DNA synthesis was performed using primers and probe sequences specific for GFP: forward primer CAACAGCCACAACGTCTATATC AT, reverse primer ATGTTGTGGCGGATCTTGAAG, Probe 5'-FAM-CCGACAAGC AGAAGAACGGCATCAA-3'TAMRA.

The viral vectors were first treated with DNaseI to remove contaminating GFP-encoding DNA. DNase buffer and DNaseI (Promega) were added to eppendorf tubes containing viral vectors (70 units of DNaseI/ml) and incubated at 37 °C for 2 hours. 10^5 cells were then infected in six-well plates in triplicate. Six hours after infection, total DNA was extracted (DNeasy kit, Qiagen, U.K.) from two samples. The third sample was incubated for a further 48 hours and then subjected to FACS analysis to enumerate infected cells. The DNA concentrations of all samples were measured using a NanoDrop ND-1000 spectro-photometer (NanoDrop Technologies, U.S.A.). 2µl of sample was then subjected to TaqMan quantitative PCR as described (Towers *et al.*, 1999) using TaqMan 2X quantitative PCR buffer (Applied Biosystems) with primers and probe at 300 nM and 150 nM, respectively. All TaqMan PCR reactions contained 0.1mg/ml salmon sperm DNA (Sigma, Poole U.K.) as a carrier.

The PCR was executed using an ABI Prism 7000 (Applied Biosystems) using the following hot start cycling parameters.

1. 10 min 95 °C
2. 15 seconds 95 °C
3. 1 min 60 °C
4. Steps 2-3 an additional 50 times

The data was analysed using ABI prism 7000 SDS software CTS 1.0 (Applied Biosystems). A standard curve was generated by preparing a 10-fold serial dilution of plasmid DNA encoding GFP (CNCG) from 5.10^5 to 50 molecules per reaction. Using the standard curve in Figure 2.3 the absolute copy numbers of GFP encoding DNA molecules present in the PCR reaction could be determined. These values were then corrected using the known concentration of the DNA sample, to give a value for the DNA copy number per 100 ng of total DNA. All PCR reactions were carried out in duplicate and the data was only accepted if no GFP target was detected in the blank controls or in DNA extracted from uninfected cells.

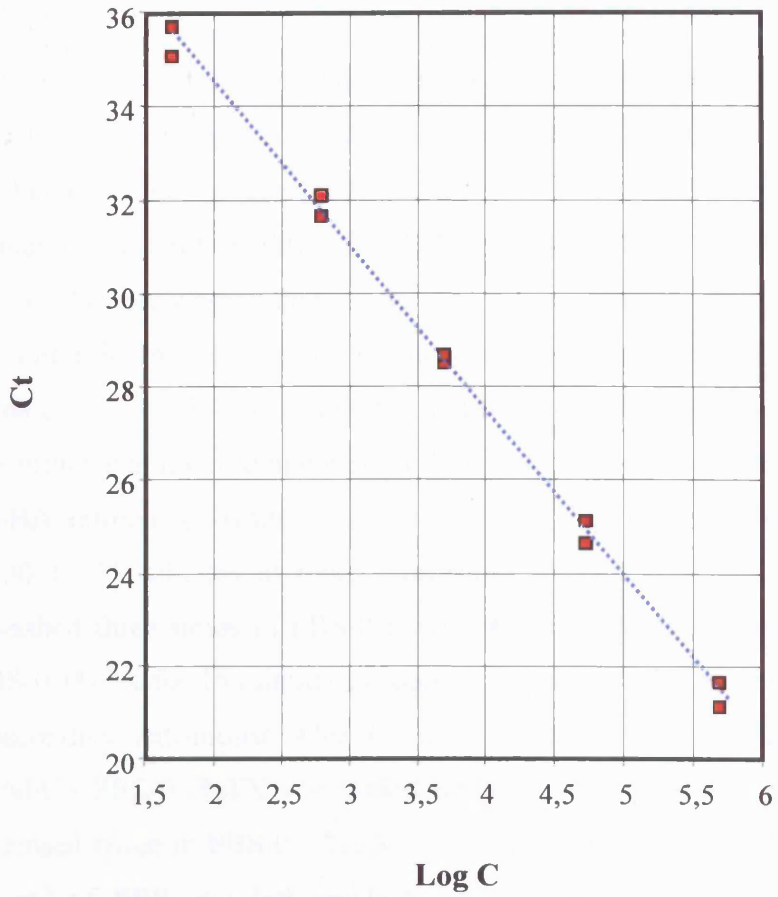


Figure 2.3. Standard curve. The standard curve for the data presented in figure 3.5. R^2 : 0.998408. Generated by ABI prism 7000 CTS 1.0 software.

2.2.8 Flow cytometry

48-hours post-infection, cells were trypsinised (trypsin-EDTA, Invitrogen, U.K.) for 2 minutes at 37 °C, resuspended in 300-500 µl of phosphate-buffered saline (PBS), transferred to FACS tubes (Falcon) and kept on ice, prior to analysis.

Green cells were then enumerated by fluorescence-activated cell sorting (FACS) analysis using a Facs Calibur or LSR (Becton Dickinson, U.K.) using Cellquest software (Becton Dickinson, U.K.). Intact cells were identified based on light scatter profiles, and only these were included in the analysis. GFP fluorescence was recorded in the FL1 channel and red fluorescence in the FL2 channel. For double-fluorescence recordings, compensation was performed using the relevant controls. A total of 10^4 to $5x10^4$ cells were analysed per sample.

2.2.9 Confocal microscopy

10^5 cells were seeded into 35mm Glass Bottom Culture Dishes of diameter (MatTek Corporation, USA). In the case of the arsenic experiments, cells were treated the next day with 8 μM As_2O_3 for the indicated periods. Cells were then washed in PBS and fixed in freshly prepared 3% paraformaldehyde (PFA, Sigma) in PBS for 30 minutes at room temperature. Cells were washed three times for 5 minutes in PBS and quenched in 50mM ammonium chloride in PBS for 15 minutes at room temperature. Cells were again washed three times in PBS for 5 minutes and incubated in 2%FCS-PBS-0.1%TritonX (TX) for 15 minutes at room temperature to block non-specific binding. 50 μl of primary mouse anti-HA antibody (Covance, U.S.A.) diluted 1:1000 in 2%FCS-PBS-0.1%TX was added for 30 to 45 minutes at room temperature. Cells were rinsed twice in PBS-0.1%TX, washed three times in PBS-0.1 %TX for 5 minutes and incubated further in 2%FCS-PBS-0.1%TX for 15 minutes at room temperature to block non-specific binding. 50 μl of secondary anti-mouse AlexaFluor 488 antibody (Molecular Probes) diluted 1:500 in 2%FCS-PBS-0.1%TX was added for 30 to 45 minutes at room temperature. Cells were rinsed twice in PBS-0.1 %TX and washed four times in PBS-0.1 %TX for 5 minutes. 1 ml of PBS was left inside the dish and cells were ready for confocal microscopy.

Microscopy analysis was executed with a Leica TCS NT Confocal Microscope (Leica Microsystems) using the Leica TCS software. The secondary antibody conjugated to photostable, green-fluorescent AlexaFluor 488 was visualised after excitation with 488 nm light for each sample. No image enhancement was used. The images in figure 3.23 are magnified 63 times.

2.2.10 Western Blot

10^6 cells were plated in 10 cm culture dishes (Helena Biosciences, U.K.). The following day, cells were incubated with the indicated concentrations of As_2O_3 for the indicated time period. The experiment was designed so that all the incubations end at the same time. Cells were then trypsinised, resuspended in PBS and counted with a haemocytometer (Hawksley BS., U.K.). Equal numbers were pelleted by centrifugation at 1200 rpm for 5 minutes. The pellet was resuspended in 300 μl of RIPA lysis buffer (0.15 M NaCl, 50 mM Tris-pH 8.0, 1% NP-40, 0.1% SDS, 0.5% deoxycholate) with protease inhibitors, according to the manufacturer's instructions (Protease inhibitor Mini

cocktail, Roche), and lysed for 30 minutes on ice. The lysate was centrifuged in a table-top microcentrifuge at 13,000 rpm for 3 minutes. The supernatants were transferred into clean, labelled eppendorf tubes and small aliquots were subjected to Bradford protein assay (Bio-Rad, according to the manufacturer's protocol). This allowed equalisation of samples by protein content. Samples were frozen at -20°C for subsequent SDS-PAGE electrophoresis and immunoblotting.

Gels were prepared using ProtoGel reagents according to the manufacturer's protocol (National Diagnostics, U.S.). Small aliquots of samples were mixed with 2xLaemli Sample buffer (125 mM Tris-HCl (pH 6.8), 1.4% SDS, 20% glycerol, 0.001% bromophenol blue, where 200 µl of β-mercaptoethanol/ml was added on the day of the experiment), boiled for 5 minutes, centrifuged at 13,000 rpm for 3 minutes and 100 ng of protein was loaded onto the gel. Kaleidoscope protein standard (Bio-Rad) was loaded as a control. Gels were electrophoresed at 50 Volts per gel (variable Amps). Electrophoresed proteins were transferred to a PVDF membrane using a Hoefer Semiphor transfer apparatus at 0.8 mA/cm^2 transferable area, for 45-60 minutes. This was sufficient for complete transfer of 55-60 kDa proteins. Blocking of non-specific binding sites on the PVDF membrane was performed in a solution of 5% non-fat milk powder in 0.1% Tween20 in Tris-buffered saline (TBS). Membranes were incubated for 1 hour with primary anti-HA, anti-cyclophilin, anti-β-actin, or anti-HDAC-1 antibodies (see table 2.3), washed three times for 10 minutes in TBS and incubated for 45 minutes with species-specific secondary, horseradish peroxidase-linked (HRP) antibodies (Sigma). Membranes were washed again in TBS (three times for 10 minutes). Proteins were then detected by enhanced chemiluminescence (ECL, Amersham) and the immunoblots were exposed to Amersham hyperfilm.

In some cases immunoblotted, exposed membranes were later stripped by incubating membranes for 20 minutes in Stripping Buffer (0.2 M glycine in distilled water, pH 2.0) at room temperature, washed three times for 10 minutes in TBS and re-blocked in a solution of 5% non-fat milk powder in 0.1% TBS. Immunoblotting with new antibodies followed as described above. As many as 3 stripping procedures could be performed without affecting the quality of the subsequent blot.

Name	Protein Size (kDa)	Source	Description
Anti-HA-Peroxidase	detects HA-tagged TRIM5α in this study of app. 55-60 kDa in size	Roche	monoclonal rat antibody against HA peptide (YPYDVPDYA), used in this study to detect HA-tagged TRIM5α, dilution 1:500
Anti-cyclophilin A	18	Biomol	rabbit polyclonal antibody against cyclophilin A, dilution 1:10 000
Anti-actin	42	Sigma	mouse monoclonal antibody against α- or β-actin, dilution 1:5000
Anti-HDAC-1	65	Upstate Biotechnology	rabbit polyclonal antibody against histone deacetylase-1, dilution 1:1000

Table 2.4. Antibodies.

Chapter 3

Results

3.1. Results 1

3.1.1 Introduction

The cross-saturation studies and the similarities between the Ref1 and Lv1 restriction phenotype suggested that Ref1 might be a human-specific variant of Lv1 that lacks the ability to restrict HIV-1, HIV-2 and SIV_{mac}. Variability in the restriction profiles of cell lines from different primate species might be explained by the divergence of a single ancestral Lv1 gene. Alternatively, it is also possible that restriction factors could have arisen on multiple occasions and that the different restriction properties of primate cell lines reflect the action of several independent genes. Indeed, endogenous retroviral envelope-mediated Fv4-like restrictions have arisen on multiple occasions (reviewed in Stoye, 2002; Bieniasz, 2003; Towers, 2003).

The identification of TRIM5α as a molecule responsible for HIV-1 restriction in Rhesus macaque cells (Stremlau *et al.*, 2004) allowed us to resolve this issue. There are two experimental approaches to test if a certain molecule contributes to the restrictive phenotype of a cell line. We can either express the potential restriction factor in cells that naturally do not restrict, and see if they acquire the restrictive phenotype, or we can downregulate the expression of the molecule in wild type restrictive cells and test whether these cells lose the ability to restrict. In our study, we employed both of these methods. Primates exhibit significant variability in retrovirus restriction specificity. This specificity might depend on the presence of different TRIM5α alleles within these species. We therefore cloned the TRIM5α cDNA from African green monkey cells by PCR and tested whether it accounts for the characteristic restriction pattern of the cells from which it was derived. By comparing the amino-acid sequences of human-, Rhesus macaque- and Agm TRIM5α molecules we aimed to define the part of the TRIM5α protein that might be responsible for the species-specificity. Interest in the character of the TRIM5α-mediated block led us to examine this using the quantitative PCR technique. Multiple splicing of the TRIM5 primary transcript gives rise to several protein isoforms, each increasingly shorter from the C-terminus. It was previously shown that the γ splice variant of Rhesus monkey TRIM5 can act as a dominant negative to Rhesus macaque TRIM5α antiviral function (Stremlau *et al.*, 2004). To examine the contribution of other

TRIM5 splice variants to antiviral activity, we cloned the human TRIM5δ isoform. TRIM5δ is shorter than the γ variant, completely lacks the B30.2 domain sequence and due to splicing contains different amino-acids at the C-terminus than the γ isoform. We therefore examined its ability to restrict and/or its potential to modulate TRIM5α activity when coexpressed.

3.1.2 Variations of TRIM5α from different primate species

Cells from different primate species show specific differences in their restriction properties for HIV-1, SIV_{mac} and MLV. To test whether these differences in restriction patterns can be explained by genetic differences between the TRIM5α genes of the species, we PCR amplified the TRIM5α cDNAs from human (hu), Rhesus macaque monkey (mac) and African green monkey (Agm) and cloned them into the MLV retroviral vector CXCRdEcoR1. The human TRIM5α gene was amplified by PCR from a human IMAGE clone number 6160154, whilst the macaque and Agm TRIM5αs were PCR amplified from Rhesus macaque cDNA or from a cDNA library derived from African green monkey CV1 cells (for details see chapter 2.2.2). The sequence of the Agm-, human- and Rhesus macaque TRIM5α was determined by sequencing three independent clones.

An alignment of the sequences is shown in Figure 3.1. Human and Rhesus monkey sequences shared 87% sequence identity. Agm TRIM5α differed from both human and Rhesus monkey alleles at multiple positions and contained a 20 amino-acids insertion in the B30.2 domain compared to the human sequence and 18 amino-acids compared to the Rhesus macaque sequence. This insertion is a result of tandem sequence duplication.

Important structural elements in the TRIM domains, such as the cysteines and histidines of the RING and B-box 2 domains, are conserved among the primate TRIM5α proteins studied. The most dramatic interspecies variation in TRIM5α is seen in the B30.2 domain.

Figure 3.1. Sequence variations of TRIM5α from different species. Comparison of protein sequence of TRIM5α from human, Rhesus macaque (*Macacca mulatta*) and Agm (*Cercopithicus* sp.) deduced from the cDNA sequence. There are sixty amino-acid residues per full line. "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed. Residues representing V1 variable region are in blue, V2 variable region are in green and V3 variable region are in pink. Variable regions are labelled according to Song *et al.*, 2005b.

3.1.3 Expression of the human and African green monkey TRIM5α genes in permissive feline CRFK cells

The finding that the TRIM5α protein from Rhesus macaques is able to block HIV-1 infection encouraged us to examine whether human TRIM5α contributes to the block to N-MLV and EIAV replication in human cells.

We cloned the human TRIM5α-encoding cDNA into a retroviral vector that also encodes red fluorescent protein (RFP). We chose a red fluorescent protein over puro resistance or other antibiotics markers because of the poor sensitivity of some cell lines to antibiotics. Human TRIM5α, RFP-encoding MLV virus vector was prepared and used to stably transduce feline CRFK cells. These cells were chosen because they are unable to restrict any of the retroviruses tested so far, and N-MLV and B-MLV are equally infectious on this cell line. Stable clones expressing RFP and therefore TRIM5α were then infected with N-MLV or B-MLV vectors encoding GFP. Forty-eight hours later, infected cells were analysed by FACS and the percentages of GFP-positive target cells were used to generate infectious titres. The results showed that CRFK cells expressing human TRIM5α are approximately 150-fold less permissive for N-MLV GFP than untransduced cells (Figure 3.2 A, B). The titre of B-MLV GFP remained almost unchanged in both control and transduced feline cells (Figure 3.2 C, D).

We performed a similar analysis to test the restriction properties of Agm TRIM5α and to examine its contribution to the Lv1 phenotype in Agm cells. African green monkey cells are known to restrict N-MLV, HIV-1, HIV-2 and SIV_{mac}. Upon the introduction of Agm TRIM5α into CRFK cells we observed 15-25-fold lower infectivity of HIV-2 GFP, N-MLV GFP and SIV_{mac} GFP, and 6-fold lower titre of HIV-1 GFP while B-MLV GFP infectivity was unaffected (Figure 3.3).

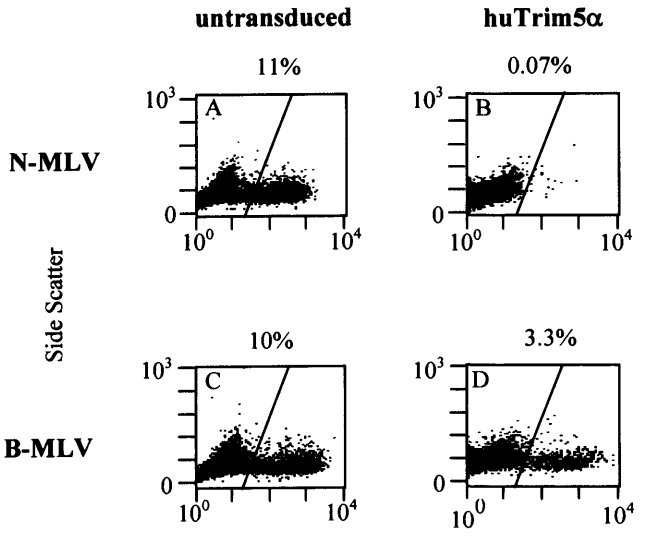


Figure 3.2. Expression of human TRIM5α in cat cells enables them to restrict N-MLV but not B-MLV. Cat CRFK cells were transduced with a retroviral vector encoding human TRIM5α and single-cell clones were isolated. Untransduced CRFK cells (*A, C*) and TRIM5α-positive clones (*B, D*) were infected with equivalent doses of N-MLV (*A, B*) or B-MLV (*C, D*) encoding GFP. The multiplicity of infection for unmodified CRFK cells was 0.1. Forty-eight hours later, the cells were analysed for green fluorescence by FACS. Side scatter is shown on the *y* axis, and GFP or MLV infectivity is shown on the *x* axis. Percentages given indicate the proportion of GFP-positive cells (MLV infected). Data shown are representative of experiments performed on 12 independent TRIM5α -positive CRFK clones.

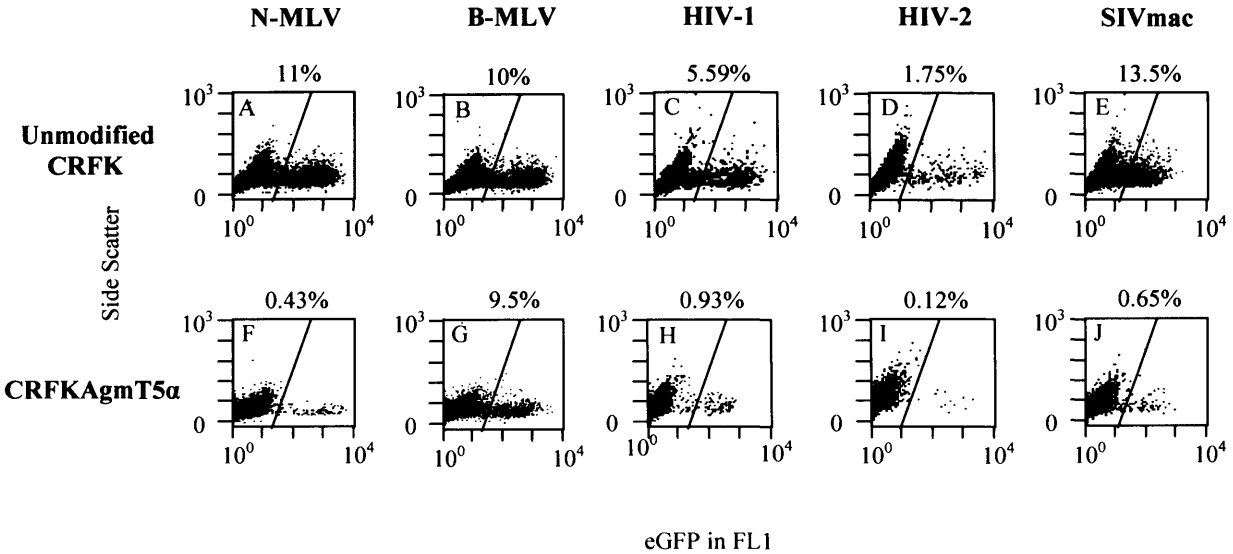


Figure 3.3. Expression of Agm TRIM5α in cat cells enables them to restrict various retroviruses. Cat CRFK cells were transduced with a retroviral vector encoding TRIM5α from Agm CV1 cells, and single-cell clones were isolated. Untransduced CRFK (*A-E*) cells and Agm TRIM5α -positive clones (*F-J*) were infected with N-MLV (*A, F*), B-MLV (*B, G*), HIV-1 (*C, H*), HIV-2 (*D, I*) or SIV_{mac} (*E, J*) encoding GFP. The multiplicity of infection was between 0.01 and 0.1. Forty-eight hours later, the cells were analysed for green fluorescence by FACS. Side scatter is shown on the *y* axis, and green fluorescence is shown on the *x* axis. Percentages given indicate the proportion of GFP-positive cells (virus infected). Data shown are representative of experiments performed on 7 independent Agm TRIM5α -positive CRFK clones.

3.1.4 Disruption of TRIM5α expression in human and Agm cells

To further confirm the identity of human TRIM5α as Ref1, we transiently downregulated its expression in human HeLa cells by introducing short interfering RNAs (siRNA) directed against human TRIM5α. These siRNAs were previously shown to significantly decrease Rhesus macaque TRIM5α protein levels (Stremlau *et al.*, 2004). This treatment had no effect on the HeLa titre of B-MLV GFP but increased that of N-MLV GFP by around 50-fold, almost up to the level of B-MLV GFP. Untransduced cells were used as a control (Figure 3.4 A).

Similarly, to confirm that Agm TRIM5α is the factor responsible for Lv1 activity, and responsible for restricting N-MLV GFP, HIV-1 GFP , SIV_{mac} GFP and EIAV GFP, in African green monkey CV1 cells, we transiently downregulated expression of Agm TRIM5α in these cells. Disruption of Agm TRIM5α expression in Agm CV1 cells rescued permissivity to restricted virus by 1- to 2- orders of magnitude without significantly affecting the titre of unrestricted B-MLV GFP (Figure 3.4 B).

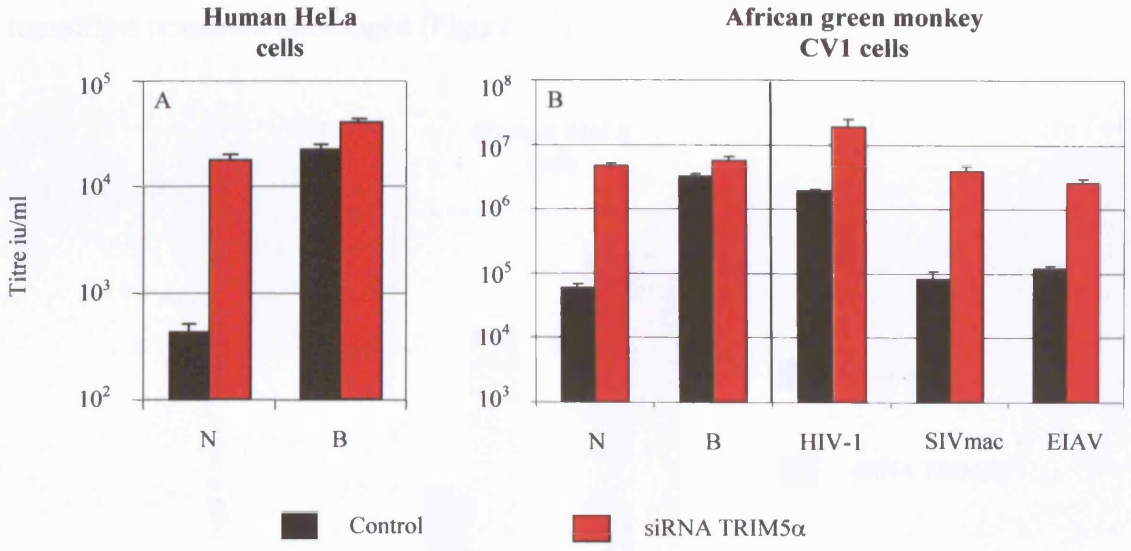


Figure 3.4. Disruption of TRIM5α expression by siRNA in human or Agm cells increases permissivity for restricted virus but not unrestricted virus. Human HeLa cells (*A*) or Agm CV1 cells (*B*) were transfected with siRNA to TRIM5α (red bars) or left untransfected as a control (black bars). Infectious titres of restricted and nonrestricted viruses were determined by infection followed by assay of GFP by FACS 48 h later. Infections were performed such that between 1% and 3% of wild type target cells were infected. Results are representative of three independent experiments.

3.1.5 Examining the impact of TRIM5α on MLV reverse transcription

The block to N-MLV infection in human cells was shown to be early post-entry, before reverse transcription (Towers *et al.*, 2000). Measuring the levels of reverse transcription DNA products of restricted N-MLV, and unrestricted B-MLV, in human cells by PCR showed that reverse transcription of N-MLV is less efficient than reverse transcription of B-MLV. Thus, relieving the block to N-MLV infection by disruption of TRIM5α expression should also rescue the ability of N-MLV to reverse transcribe.

We therefore compared the levels of viral DNA synthesis of restricted and nonrestricted virus in human HeLa cells in the presence or absence of human TRIM5α. Viral DNA was measured 6 hours post-infection by TaqMan quantitative PCR using primer and probe sequences specific for GFP. To exclude the possibility of contamination by GFP-encoding DNA from the virus preparation, all viral vectors were treated with DNaseI before infection. In the wild type cells, N-MLV synthesised between 10-100-fold less viral DNA than B-MLV. In the cells where the expression of human TRIM5α was downregulated by siRNA, N-MLV was able to synthesise approximately 10 times more viral DNA than in control unmodified cells, whereas the level of B-MLV reverse transcripts remained unchanged (Figure 3.5).

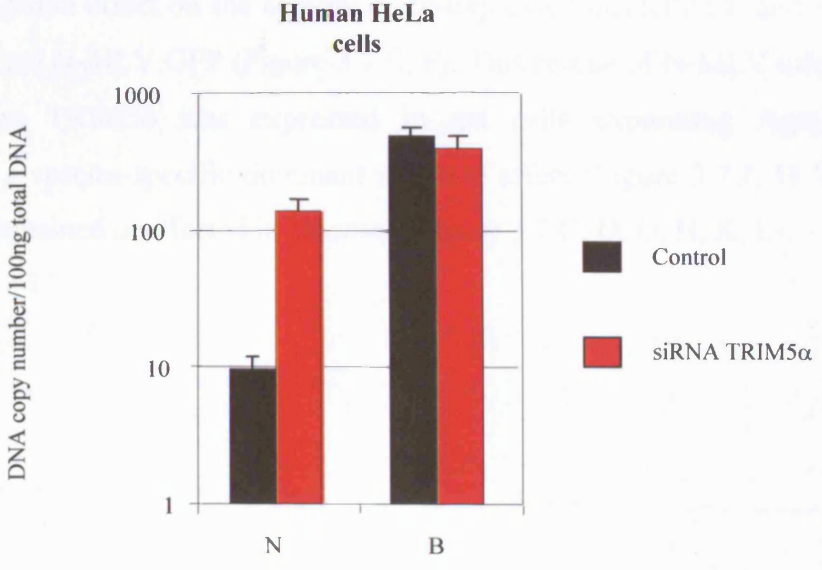


Figure 3.5. Disruption of TRIM5α expression by siRNA in human cells increases N-MLV but not B-MLV viral DNA synthesis. Human HeLa cells were transfected with siRNA to TRIM5α (red bars) or left untransfected as control (black bars) and infected with equivalent doses of N-MLV and B-MLV encoding GFP. Six hours after infection total DNA was purified and subjected to TaqMan quantitative PCR as described in *Materials and Methods*. Viral DNA copy number per 100 ng of total DNA is plotted. Results are representative of two independent experiments.

3.1.6 Investigating the role of the TRIM5delta splice variant in restriction

The human TRIM5δ gene was amplified by PCR from a plasmid kindly provided by P.G. Pelicci and cloned into a retroviral vector expressing red fluorescent protein. Cloning of human TRIM5δ allowed us to test its restriction capability and its contribution to TRIM5α–mediated restriction. We stably transduced human TE671 cells with a Moloney retroviral vector encoding RFP and human TRIM5δ, and challenged these cells with N-MLV or B-MLV vectors encoding GFP. Overexpression of TRIM5δ did not enhance the block by TE671 cells to N-MLV. On the contrary, it increased the permissivity to N-MLV infection by almost 60-fold, demonstrating that TRIM5δ exerts dominant negative activity on TRIM5α function (Figure 3.6 A, B). Unrestricted B-MLV infectivity was unaffected (Figure 3.6 C, D).

We were also interested to see if human TRIM5δ has dominant negative activity on TRIM5α from species other than human. We therefore transiently expressed human TRIM5δ in cat cells stably over-expressing human TRIM5α or Agm TRIM5α. Introduction of human TRIM5δ into wild type feline CRFK cells did not impact on permissivity to MLV infection (Figure 3.7 A, B) thus confirming the importance of the B30.2 domain in restriction. However, transient expression of huTRIM5δ in cat cells had a dominant negative effect on the activity of co-expressed huTRIM5α, and relieved the block to restricted N-MLV GFP (Figure 3.7 E, F). This rescue of N-MLV infectivity was not seen when TRIM5δ was expressed in cat cells expressing Agm TRIM5α, demonstrating a species-specific dominant negative effect (Figure 3.7 I, J). The titre of B-MLV GFP remained unaffected in all cases (Figure 3.7 C, D, G, H, K, L).

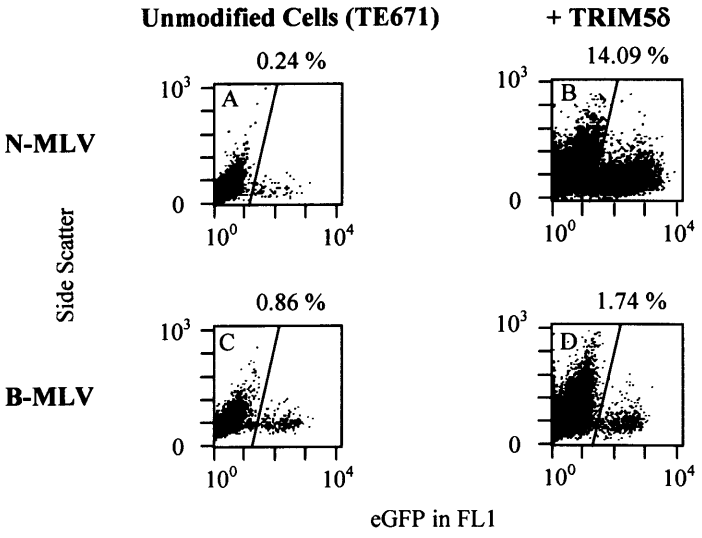


Figure 3.6. Human TE671 cells stably over-expressing the human TRIM5δ isoform have increased permissivity to N-MLV but not B-MLV infection. TE671 cells were transduced by a retroviral vector encoding human TRIM5δ and single-cell clones were isolated. Wild type TE671 cells (*A* and *C*) and red fluorescent protein expressing TRIM5δ-positive clones (*B* and *D*) were infected with N-MLV (*A, B*) or B-MLV (*C, D*) encoding GFP. Forty-eight hours later, the cells were analysed for green fluorescence by FACS. Percentages given indicate the proportion of GFP-positive cells (virus infected). Infections were performed such that around 0.5% of wild type target cells were infected. Data shown are representative of experiments performed on 3 independent TRIM5δ -positive TE671 clones.

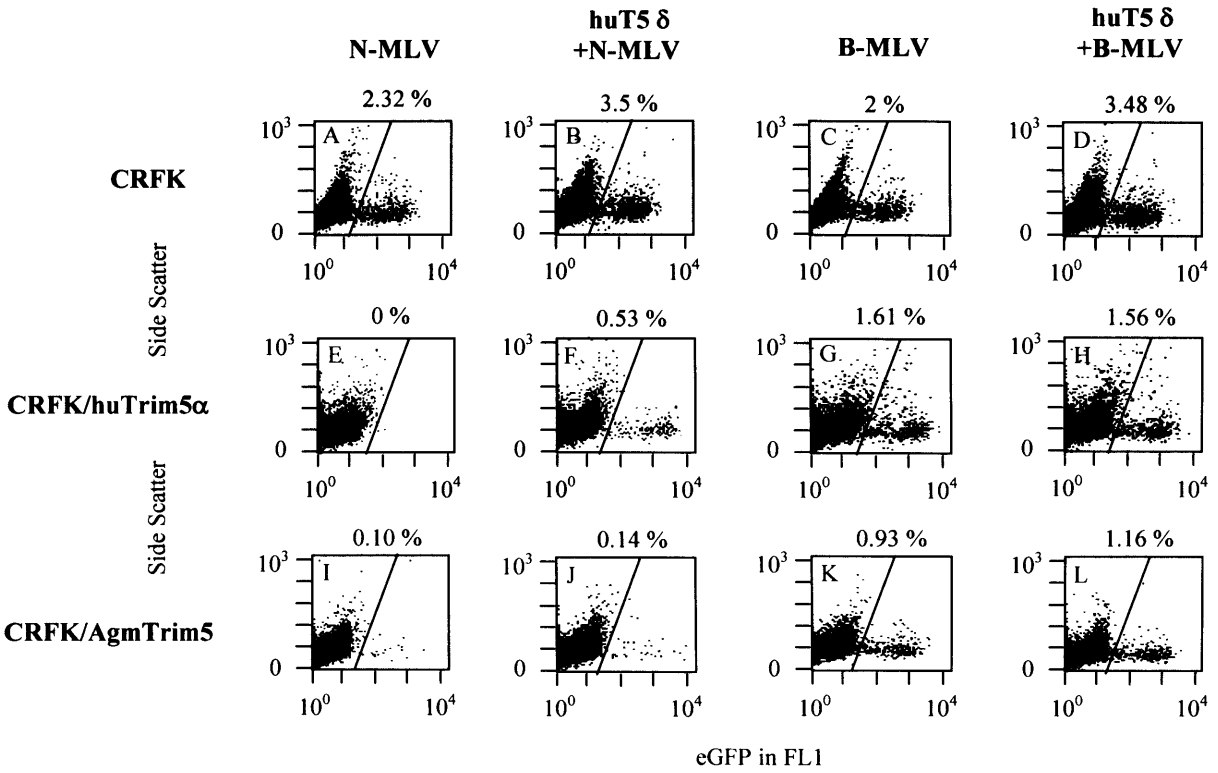


Figure 3.7. The dominant negative effect of human TRIM5δ is species specific. We infected unmodified cat CRFK (*A-D*) or cat cells stably over-expressing either human TRIM5α (*E-H*) or Agm TRIM5α (*I-L*) with N-MLV GFP (*A, B, E, F, I, J*) or B-MLV GFP (*C, D, G, H, K, L*) in the presence (*B, D, F, H, J, L*) or absence (*A, C, E, G, I, K*) of human TRIM5δ isoform. 48 hours later cells were analysed by FACS. Infections were performed such that around 2% of untransduced target cells were infected. Results shown are representative of 3 independent experiments.

3.1.7 Discussion

Intracellular antiviral responses are one of the defense mechanisms employed by host organisms against retroviral pathogens. Although evidence of specific innate cellular factors that inhibit retroviral replication was first provided by studies of MLV replication in mice (Lilly *et al.*, 1967; Lilly and Pincus, 1973) it later became evident that primate species including humans also possess these innate anti-retroviral activities (Besnier *et al.*, 2002; Cowan *et al.*, 2002; Munk *et al.*, 2002; Towers *et al.*, 2002).

Human cells were shown to block the infection of N-tropic MLV and of the distantly related equine retrovirus EIAV. Monkey cells displayed a broader range of restrictive activities, and blocked viruses like HIV-1, HIV-2, N-MLV, EIAV and certain types of SIV (Towers *et al.*, 2000, 2002; Besnier *et al.*, 2002; Hatziioannou *et al.*, 2003). The factor responsible for this block was termed Ref1 in human cells and Lv1 in monkey cells (Cowan *et al.*, 2002; Towers *et al.*, 2002). It was not known if Ref1 and Lv1 were two independent restrictive mechanisms responsible for retroviral blocks in humans and monkeys or if they are related. An increasing amount of circumstantial evidence suggested that Ref1 and Lv1 are related. Both restriction factors are saturated with high doses of restricted virus, they both act before reverse transcription, are able to restrict divergent mammalian retroviruses and both distinguish between very closely related N-tropic and B-tropic MLV (Towers *et al.*, 2000; Besnier *et al.*, 2002; Cowan *et al.*, 2002; Munk *et al.*, 2002; Towers *et al.*, 2002; Hatziioannou *et al.*, 2003). The discovery of TRIM5α restriction factor in Rhesus macaque monkey cells (Stremlau *et al.*, 2004) made it possible to examine whether TRIM5α encodes Ref1 and Lv1 activity.

By over-expression of human and African green monkey TRIM5α in otherwise nonrestricting feline cells (Figure 3.2 and 3.3), and their depletion from wild type cells (Figure 3.4), we showed that TRIM5α is responsible for both Ref1 activity in humans and Lv1 activity in simians. In Figure 3.2 we showed that expression of human TRIM5α in nonrestricting cat cells renders them able to restrict N-MLV but not the very closely related B-MLV. The degree of restriction was over 2 orders of magnitude higher than when not expressed and comparable with restriction of N-MLV in human cell lines. We also observed a slight (3-fold) block to B-MLV infection in cat cells expressing human TRIM5α. This is likely to be due to over-expression of human TRIM5α. It was shown previously that the over-expression of the murine restriction factor $Fv1^b$ expanded its specificity, leading to a weak restriction of B-MLV (Bock *et al.*, 2000). The ability of human TRIM5α to restrict retroviral infection is further confirmed in Figure 3.4, where

disruption of human TRIM5α expression restored the infectivity of restricted N-MLV in nonpermissive human HeLa cells to titres equivalent to unrestricted B-MLV. The titre of B-MLV is only slightly affected.

We performed analogous experiments to test if TRIM5α is also responsible for the restrictive properties of monkey species other than Rhesus monkey. We tested the restrictive properties of African green tantalus monkey TRIM5α from CV1 cells. African green monkeys were shown to restrict a broad range of retroviruses (Table 1.4). Our data showed that expression of Agm TRIM5α in nonrestricting cat cells confers on them the ability to restrict N-MLV, HIV-1, HIV-2 and SIV_{mac}, but not B-MLV (Figure 3.3). Furthermore, stable down-regulation of Agm TRIM5 in Agm CV1 cells restores infectivity to a range of restricted viruses, including N-MLV, HIV-1, SIV_{mac} and EIAV (Figure 3.4 B). The titre of N-MLV is restored to that of B-MLV (Figure 3.4 B). The titre of restricted viruses increased in concordance with the strength of the blocks, as defined by titration of these viruses on nonrestricted feline cells.

Thus, we demonstrated that human- and Agm TRIM5α can potently, and specifically, block infection of many unrelated retroviruses, and that Ref1 and Lv1 are species-specific variants of a single restriction factor. TRIM5α is both necessary and sufficient for the establishment of the specific restriction of retroviral infection in human, Agm and feline cell lines expressing these proteins. The results also indicate that any cofactors required for the block to retrovirus replication must be functionally conserved between humans, cats and monkeys.

It has also become clear that primate TRIM5α orthologues inhibit retrovirus replication in a species-specific and virus-specific manner. Human cells were shown to restrict N-MLV and EIAV but not B-MLV, HIV-1, or SIV_{mac}, while Rhesus macaque cells are able to restrict HIV-1, HIV-2 and SIV_{Agm} but not MLV viruses. African green monkey cell lines exhibit the broadest range of restricted retroviruses, namely N-MLV, HIV-1, HIV-2, SIV_{mac} and EIAV, although this apparent broader specificity is likely to be a consequence of the range of viruses tested. As we have shown, expression of TRIM5α from a certain species in cat cells bestows on them the same restrictive properties as the cell line from which the TRIM5α was derived. Therefore, it appears that the observed specificity for particular retroviruses arises from differences in the TRIM5α proteins themselves.

In Figure 3.1 we compared the amino-acid sequences of TRIM5α alleles from human TE671, Rhesus macaque monkey FRhK and African green monkey CV1 cells, and showed that considerable variation among TRIM5α proteins of these primate species

exist. Human TRIM5α and Rhesus macaque TRIM5α exhibit approximately a 13% variation in amino-acid sequence. Agm TRIM5α differed from both human and Rhesus monkey variants at multiple positions and, as a result of tandem sequence duplication, contained an insertion in the B30.2 domain (relative to the human and Rhesus sequence). Key elements of TRIM domains, such as the cysteines and histidines of the RING and B-box 2 domains, are preserved among the primate TRIM5α proteins studied. The other sequences of the RING, B-box 2 and coiled coil domains share many similarities. The most dramatic variation among primate TRIM5α proteins occurs in the B30.2 domain, suggesting that this domain is under the most selective pressure and might be responsible for the differential retroviral restriction capabilities of different TRIM5α's. It may therefore be possible that the 20 amino-acid insertion in the B30.2 domain of Agm TRIM5α, compared to the human one, might be responsible for the broader range of targeted viruses by Agm TRIM5α. Indeed, this was examined in the recent publication of Nakayama *et al.*, where it was shown that mutant Agm TRIM5α lacking the 20 amino-acid duplication completely lost the ability to restrict SIV_{mac} infection while still retaining the ability to restrict HIV-1 (Nakayama *et al.*, 2005). These results, together with finding of other groups performing mutagenesis studies on simian and human TRIM5α, clearly indicated that the B30.2 domain contains a determinant of species-specific restriction (Nakayama *et al.*, 2005; Perez-Caballero *et al.*, 2005a; Sawyer *et al.*, 2005; Stremlau *et al.*, 2005; Yap *et al.*, 2005).

In Figure 3.5 we examined the levels of N-MLV reverse transcription in the presence or absence of human TRIM5α. Our results showed that reverse transcription of N-MLV in human cells is rescued in the absence of human TRIM5α and thus it is TRIM5α that is responsible for the block of N-MLV before reverse transcription. Levels of viral DNA of unrestricted B-MLV were unchanged in both cases, in the presence or absence of endogenous TRIM5α. However, it is important to point out that the magnitude of the block to DNA does not necessarily account for the block to infection. Virus can be restricted in more than one step of its life cycle. Wu *et al.*, (2006) showed that this is the case for TRIM5α-mediated restriction. This study revealed that even when you let TRIM5α-sensitive virus efficiently reverse transcribe, it is still restricted by TRIM5α at a later post-RT step.

Investigating the involvement of other TRIM5 variants in retroviral restriction showed that the δ splice variant of TRIM5 lacking the C-terminal domain lost its restriction activity. This further confirms the importance of the C-terminal domain for restriction (compare Figure 3.7 A and B). TRIM5δ exerts a dominant-negative effect on wild type

TRIM5α when these two proteins are co-expressed, and this effect is seen only in the species-specific context (Figure 3.6 and 3.7). It is likely that TRIM5α proteins might homo-multimerise through their coiled-coil domain in order to make a functional antiviral complex. As the δ isoform possesses the same coiled-coil domain, its dominant negative effect might simply be explained by the disruption of TRIM5α homomultimers when expressed at sufficient levels, which might cause improper creation of functional antiviral TRIM5α complexes. In concordance with this is the recent report that the TRIM5α protein oligomerises into trimers, and that trimerisation is needed for the B30.2 domains to interact with threefold pseudo-symmetrical structures present on retroviral capsids (Mische *et al.*, 2005). Thus, truncated TRIM5α proteins lacking B30.2 domains, but maintaining the coiled-coil domain, form heteromultimers with full-length TRIM5α, and are dominant inhibitors of its function (Javanbakht *et al.*, 2005; Perez-Caballero *et al.*, 2005a). It is not only truncated TRIM5α proteins that can act in a dominant negative way. Expression of full-length Rhesus monkey TRIM5α, or Owl monkey TRIMCyp, in human cells interfered with the anti-N-MLV activity of endogenous human TRIM5α (Berthoux *et al.*, 2005), and TRIM5α from cynomolgus monkey had a dominant-negative effect on the anti-SIV_{mac} activity of Agm TRIM5α (Nakayama *et al.*, 2006). The dominant negative effect of human TRIM5δ was only seen on human TRIM5α and not Agm TRIM5α (Figure 3.7). Similarly, overexpression of human TRIM5δ in Agm CV1 cells did not exert a dominant negative effect on endogenous Agm TRIM5α (L.J. Ylinen, unpublished results). This could be explained by human TRIM5δ not being able to form multimers with Agm TRIM5α, or that the multimers created between human TRIM5δ and Agm TRIM5α are tolerated and do not inhibit the restrictive properties of the α molecules. The presence and conservation of truncated TRIM5 products within the genomes suggests that it might serve some, perhaps regulatory, function in TRIM5α-mediated activity.

3.2 Results 2

3.2.1 Introduction

The aim of the study described in this chapter is to understand the relationship between cyclophilin A and host factors impacting on permissivity to HIV-1 in mammalian cells. Cyclophilin A is recruited into nascent HIV-1 virions, as well as incoming HIV-1 capsids, through the binding to HIV-1 capsid protein, where it isomerises an exposed proline residue (Franke *et al.*, 1994; Thali *et al.*, 1994; Ott *et al.*, 1995; Gamble *et al.*, 1996; Gitti et al., 1996; Yoo *et al.*, 1997; Bosco *et al.*, 2002; Bosco and Kern, 2004). CypA influences an early, post-entry step of HIV-1 replication in the target cell. Inhibition of CypA's activity with CSA changes the infectivity of HIV-1 in both a cell line and species-specific way. This is exemplified by the fact that the HIV-1 titre is decreased in human cells but increased in simian cells following CSA treatment. Thus, CypA has been proposed to prevent binding of restriction factor to viral capsid in human cells, resulting in enhancement of HIV-1 infectivity, and also to potentiate restriction of HIV-1 in monkey cells (Towers *et al.*, 2003). Several lines of evidence agree that the activity of CypA and TRIM5α may be linked: i) the viral determinants for restriction by TRIM5α map to the CypA-binding loop in the capsid protein (Ylinen *et al.*, 2005), ii) the TRIM5α block to HIV-1 generally occurs prior to reverse transcription, and this is the point in the retrovirus life cycle where CypA acts, and iii) the isolation of a restriction factor from Owl monkey cells showed it to be a fusion between TRIM5 and cyclophilin A. We therefore sought a connection between CypA and TRIM5α in cells where HIV-infectivity is influenced by CypA, but where TRIM5α is not fused to CypA.

We set out to examine the effect of CypA on HIV-1 infectivity using cells from two Old World monkey species, Rhesus macaque and African green monkey, by either treating the cells with CSA or stably downregulating cyclophilin A levels using shRNA. By stable downregulation of TRIM5α, we wanted to examine the dependence of the cyclophilin A effect on the presence of a TRIM5α. Further, we wanted to test if expression of simian TRIM5 in permissive feline cells renders them able to restrict HIV-1 in a cyclosporine A-sensitive way. TRIMCyp recruits its tripartite motif to HIV-1 capsid via cyclophilin A and, therefore, its ability to restrict completely depends on the ability of the virus to bind CypA. HIV-1 mutated in its CypA-binding loop is thus protected from the TRIMCyp restriction activity. By using CypA-binding loop mutants,

we wanted to examine whether cyclophilin A has the same recruitment role in the case of Old World monkey TRIM5α. Furthermore, we extended this investigation to human cells to learn more about the involvement of cyclophilin A and TRIM5α in permissivity of human cells to HIV-1 infection.

3.2.2 Cyclophilin A has anti-HIV-1 activity in Old World monkey cell lines

We examined the effect of cyclophilin A on HIV-1 replication in two different simian cell lines, Rhesus macaque FRhK cells and African green monkey CV1 cells. We inhibited CypA enzymatic function with 5 μM CSA, or by stably downregulating CypA expression with CypA-specific shRNA. These cells were then infected with titrations of various strains of VSV-G-pseudotyped HIV-1 GFP vectors. We used NL4.3, an X-4-tropic laboratory-adapted HIV-1 strain (Adachi *et al.*, 1986). NL4.3(Ba-L) is NL4.3 encoding the CypA-binding loop from HIV-1 Ba-L (CA mutations H87Q, A88P, and I91V) and has been reported to be CypA independent and higher titre in simian cells (Kootstra *et al.*, 2003; Ikeda *et al.*, 2004). 92BR is a naturally occurring subclass C primary isolate identified in Brazil (Gao *et al.*, 1996). MVP5180 is an O group virus reported to be CypA independent in human cells (Braaten *et al.*, 1996c; Wiegers and Krausslich, 2002).

As shown in Figure 3.8, the effect of cyclophilin A on retroviral infection is species and HIV-1 sequence specific. Reduction of CypA expression had the same effect as treatment with 5 μM CSA in each case, confirming CypA as the relevant target for CSA (Figure 3.8 A to H). Infection of both, NL4.3 and 92BR strains is enhanced by around 10-fold in the absence of cyclophilin A activity on cell lines from Agm and Rhesus macaques (Figure 3.8 A, C, E, G). Infectivity of the NL4.3 (Ba-L) strain is not affected by cyclophilin A in any cell line (Figure 3.8 B, F). The influence of cyclophilin A on the infectivity of MVP differs between FRhK and CV1 cell lines. Whilst MVP titre is enhanced by around 7-fold on Agm CV1 cells when CypA activity was abrogated (Figure 3.8 D), it was not significantly increased in FRhK cells (Figure 3.8 H). These data indicate that elimination of CypA activity, either by reduction of CypA expression or by inhibition of CypA enzymatic activity, increases the infectivity of wild-type HIV-1 on Old World monkey Agm and macaque cell lines. Furthermore, mutations within HIV-1 capsid protein can render HIV-1 insensitive to CypA activity.

The efficiency of downregulation of CypA levels in FRhK and CV1 cells was confirmed by biochemical and genetic approaches. Treatment with CSA did not significantly

increase NL4.3 infectivity when CypA expression was reduced, indicating that the reduction of CypA expression by shRNA is significant (Figure 3.8 I, J). Similarly, western blot analysis revealed that CypA levels were substantially reduced, with actin expression levels shown as a control (Figure 3.8 K).

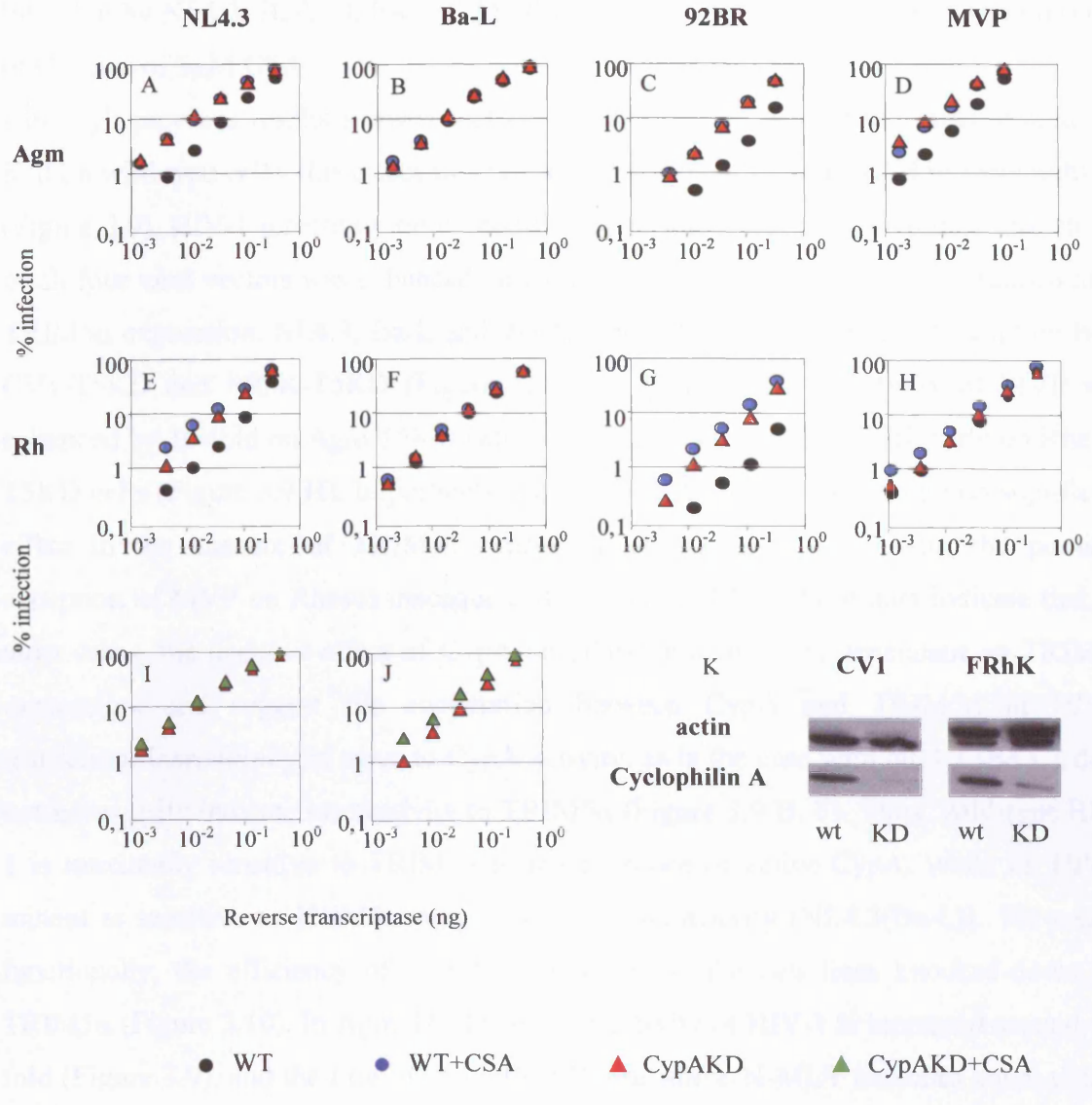


Figure 3.8. Abrogation of cyclophilin A activity rescues HIV-1 infectivity in Old World monkey cell lines. HIV-1 GFP-encoding vectors were made using Gag from NL4.3, 92BR, MVP5180, and NL4.3(Ba-L). Threefold serial dilutions were titrated onto Agm CV1 (*A to D*) and Rhesus FRhK4 (*E to H*) cells. The cells were unmodified and untreated as a control (●), stably expressed shRNA to CypA (CypAKD) (▲), or were treated with 5 μM CSA (●). NL4.3 GFP was also titrated onto Agm CV1 cells (*I*) or Rhesus FrhK4 cells (*J*) stably expressing shRNA to CypA in the presence (▲) or absence (▲) of 5 μM CSA. Virus input doses were measured in nanograms of reverse transcriptase as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus. (*K*) Western blot of extracts of CV1 and FRhK4 cells expressing reduced levels of CypA. Extracts probed for cyclophilin A from wild-type cells (wt) and cells expressing shRNA to CypA (KD) are shown. α-actin blots are shown as a control for equal loading.

3.2.3 TRIM5α is required for anti-HIV-1 activity of CypA in simian cells

We next examined the role of TRIM5α involvement in the effect of cyclophilin A on HIV-1 infection in simian cells. We stably downregulated TRIM5α expression in macaque FRhK and Agm CV1 cells using TRIM5-specific shRNA cloned into a lentiviral vector. These cells (FRhK-T5KD and CV1-T5KD) were then subjected to infection by NL4.3, Ba-L, 92BR and MVP HIV-1 vectors encoding GFP in the presence or absence of 5μM CSA.

Although previous results showed that CSA increased the titre of the virus around 10-fold on wild type cells, this effect was not seen when the levels of TRIM5α were reduced (Figure 3.9). HIV-1 infectivity on unmodified cells was included as a control. Infectivity of all four viral vectors was enhanced on Agm and Rhesus macaque cells by reduction of TRIM5α expression. NL4.3, Ba-L and 92BR were enhanced by around 10-fold on both CV1-T5KD and FRhK-T5KD (Figure 3.9 A-C, E-G), while infectivity of MVP was enhanced by 10-fold on Agm T5KD cells (Figure 3.9 D), but not significantly on Rhesus T5KD cells (Figure 3.9 H). Importantly, inhibition of CypA with CSA had no significant effect in the absence of TRIM5α expression (Figure 3.9 A-G), with the possible exception of MVP on Rhesus macaque cells (Figure 3.9 H). These data indicate that, in most cases, the negative effect of CypA on HIV-1 infectivity is dependent on TRIM5α expression, and suggest the cooperation between CypA and TRIM5α in HIV-1 restriction. Insensitivity of virus to CypA activity, as is the case with NL4.3 (Ba-L), does not necessarily indicate insensitivity to TRIM5α (Figure 3.9 B, F). Thus, wild-type HIV-1 is maximally sensitive to TRIM5α in the presence of active CypA, while an HIV-1 mutant is sensitive to TRIM5α regardless of CypA activity [NL4.3(Ba-L)]. We tested, functionally, the efficiency of TRIM5α reduction in the cell lines knocked-down for TRIM5α (Figure 3.10). In Agm T5KD cells, infectivity of HIV-1 is increased around 10-fold (Figure 3.9), and the titre of Agm TRIM5α-sensitive N-MLV becomes equal to that of B-MLV, which is TRIM5α-insensitive (Figure 3.10 B). In Rhesus macaque cells, a significant reduction in TRIM5α expression is indicated by a 10-fold increase in HIV-1 infectivity (Figure 3.9). N-MLV infectivity is also slightly increased to the level of B-MLV (Figure 3.10 D), in concordance with previous data showing weak restriction of N-MLV, but not B-MLV, by Rhesus macaque TRIM5α (Hatziioannou *et al.*, 2004b). Wild-type, untreated, Agm and Rhesus macaque cells infected with N-MLV and B-MLV are shown as a control (Figure 3.10 A, C).

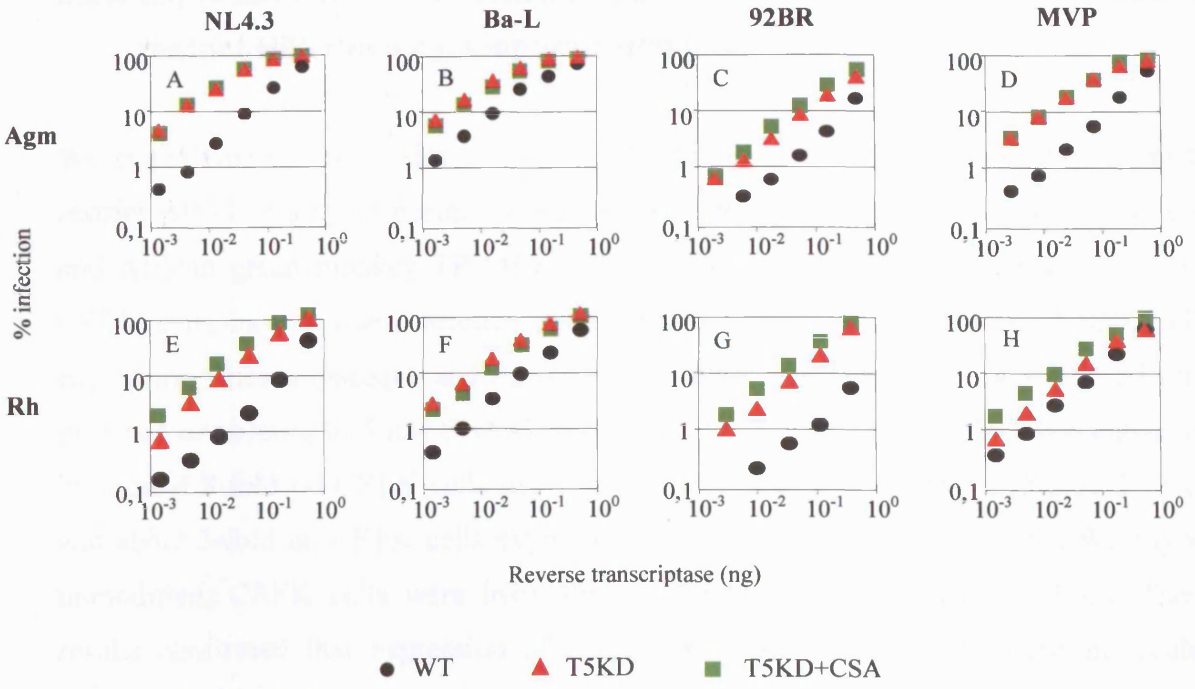


Figure 3.9. Reduction of TRIM5α protein levels renders HIV-1 infectivity insensitive to cyclosporine A in cells from Old World monkeys. HIV-1 GFP-encoding vectors were made using Gag from NL4.3, 92BR, MVP5180, or NL4.3(Ba-L). Threefold serial dilutions were titrated onto Agm CV1 cells (*A to D*) and Rhesus FRhK4 cells (*E to H*). The cells were unmodified and untreated as a control (●), stably expressed shRNA to TRIM5 (T5KD, ▲), or stably expressed shRNA to TRIM5 and treated with 5 μM CSA (■). Input doses are measured in nanograms of reverse transcriptase as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

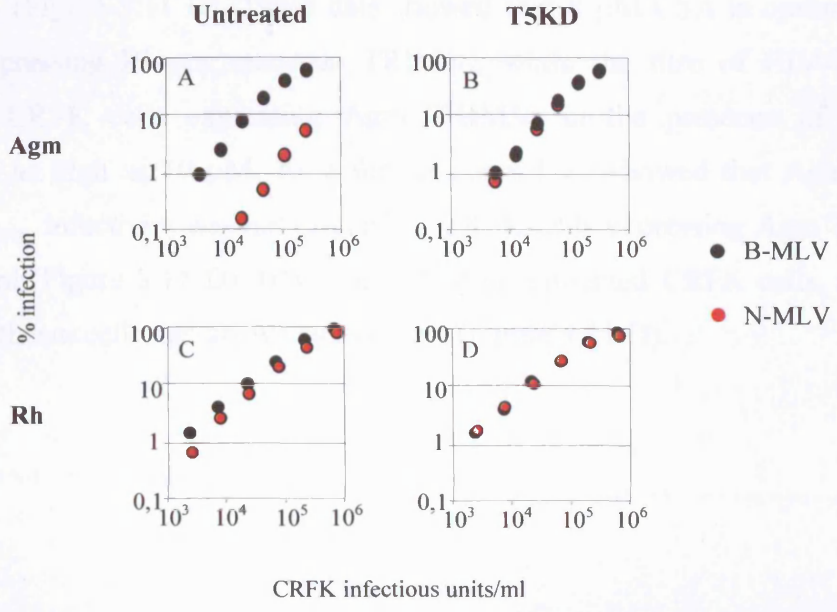


Figure 3.10. Reduction of TRIM5α protein levels renders N-MLV and B-MLV infectivity equal in simian cells. To control for the reduction of TRIM5α expression, we show that N-MLV (●) and B-MLV (●) GFP-encoding vectors have similar titres on Agm CV1 (*B*) or FRhK4 Rhesus (*D*) cells expressing reduced levels of TRIM5α (T5KD). N-MLV and B-MLV infectivities on untreated cells are shown as control Agm (*A*) and Rhesus (*C*). MLV input doses are shown as CRFK infectious units per millilitre. Results are representative of two independent experiments performed with two independent preparations of virus.

3.2.4 Expression of simian TRIM5α enables permissive feline CRFK cells to restrict HIV-1 in a cyclosporine-sensitive way

We tested whether expression of simian TRIM5α in non-simian cells enables them to restrict HIV-1 in a cyclosporine A-sensitive way. We stably expressed Rhesus macaque and African green monkey TRIM5α in feline CRFK cells. As mentioned previously, CRFK cells have a non-restrictive phenotype. Wild-type and transduced CRFK cells expressing Rhesus macaque and Agm TRIM5α were infected with NL4.3 HIV-1 in the presence or absence of 5 μM CSA (Figure 3.11). The infectivity of HIV-1 was enhanced by around 8-fold on CRFK cells expressing Rhesus macaque TRIM5α (Figure 3.11 B) and about 3-fold on CRFK cells expressing Agm TRIM5α (Figure 3.11 A). Wild type, unmodified, CRFK cells were insensitive to CSA treatment (Figure 3.11 C). These results confirmed that expression of Rhesus macaque and Agm TRIM5α molecules renders CRFK cells able to restrict HIV-1 in a cyclosporine A-sensitive way. The fact that the restoration of HIV-1 infectivity by CSA is less effective in the feline cells over-expressing TRIM5α (Figure 3.11) than in cells expressing endogenous levels of TRIM5α (Figure 3.8) might be explained by higher protein levels in the feline cells. To further test whether higher CSA concentrations are needed to rescue HIV-1 infectivity on these cells further, we measured infectivity of a fixed dose of HIV-1 in the presence of 0, 2, 4, 6, 8, 10 μM CSA (Figure 3.11 D). These data showed that 8 μM CSA is optimal in feline cells over-expressing Rhesus macaque TRIM5α, while the titre of HIV-1 gradually increases in CRFK cells expressing Agm TRIM5α in the presence of CSA at a concentration as high as 10 μM. As a further control we showed that Agm TRIM5α-sensitive SIV_{mac} infectivity was not rescued in CRFK cells expressing Agm TRIM5α by CSA treatment (Figure 3.11 D). HIV-1 infection on untreated CRFK cells, and SIV_{mac} infection on Rhesus cells, are shown as a control (Figure 3.11 D).

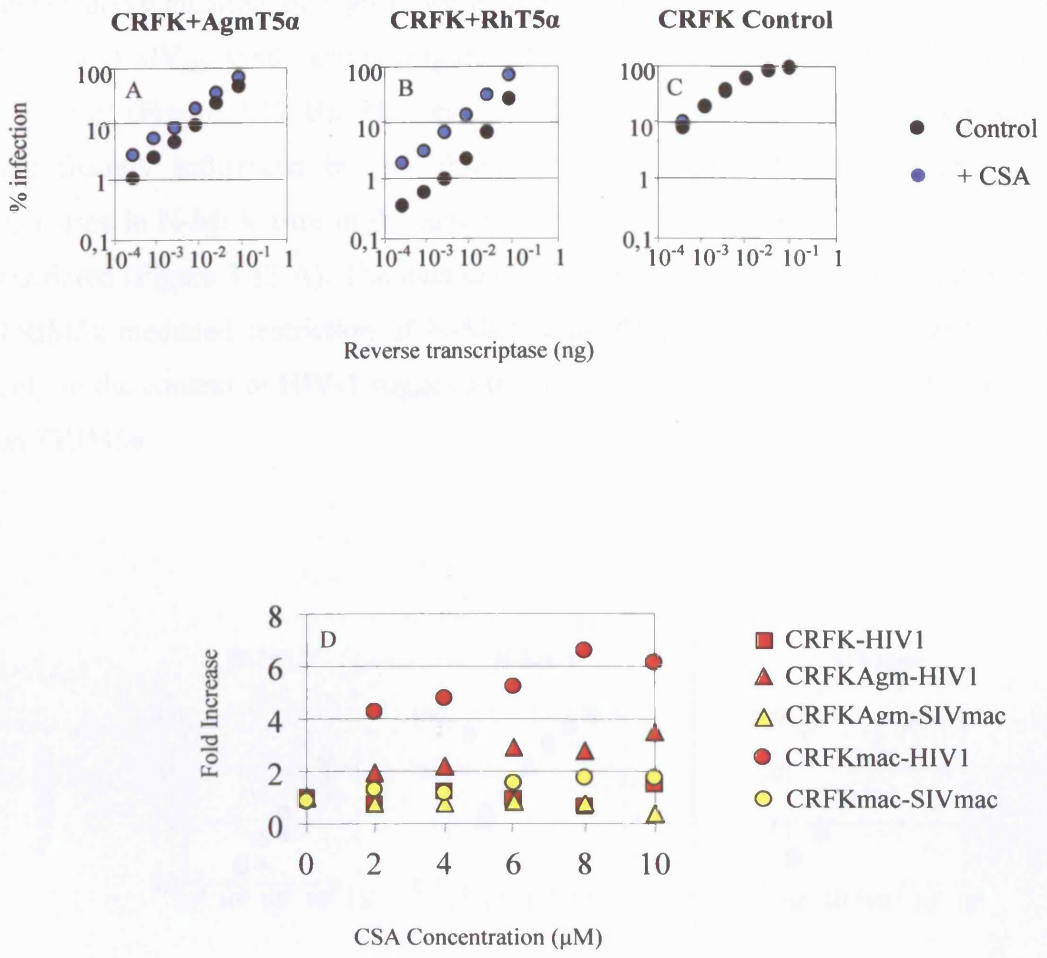


Figure 3.11. Expression of simian TRIM5α enables permissive feline CRFK cells to restrict HIV-1 in a cyclosporine-sensitive way. Serial dilutions of NL4.3 HIV-1 encoding GFP were titrated onto feline CRFK cells expressing Agm TRIM5α (*A*), Rhesus TRIM5α (*B*), or unmodified CRFK cells as a control (*C*) in the presence of 5 μM CSA (•) or left untreated as a control (•). Viral dose was measured by reverse transcriptase ELISA assay. (*D*) A fixed dose of HIV-1 (red) and SIV_{mac} (yellow), chosen to infect around 1% of the target cells, was used to infect unmodified CRFK cells (square), CRFK cells expressing Agm TRIM5α (triangles), or Rhesus TRIM5α (circles) in the presence of a serial dilution of CSA from 0 to 10 μM. The fold increase in infectivity after CSA treatment was measured and is plotted. Results are representative of two independent experiments performed with two independent virus preparations.

3.2.5 Cyclophilin A is not required for restriction of MLV or SIV_{mac} by TRIM5α

The next stage was to examine if cyclophilin A activity is required for TRIM5α-mediated restriction of retroviruses other than HIV-1. Therefore, wild type Agm CV1 cells, or CV1 cells where cyclophilin A activity was abrogated either by CSA treatment or by stable dowregulation of CypA, were infected with titrations of TRIM5α-sensitive N-MLV and SIV_{mac} viral vectors (Figure 3.12 A, C). Unrestricted B-MLV was included as a control (Figure 3.12 B). The results indicate that the titre of these viruses was not significantly influenced by the absence of cyclophilin A activity. There are small increases in N-MLV titre in the absence of CypA activity, but this virus is still strongly restricted (Figure 3.12 A). The data demonstrated that cyclophilin A is not important for TRIM5α-mediated restriction of N-MLV and SIV_{mac}. The fact that CypA is important only in the context of HIV-1 suggests that CypA has a direct effect on HIV-1, rather than on TRIM5α.

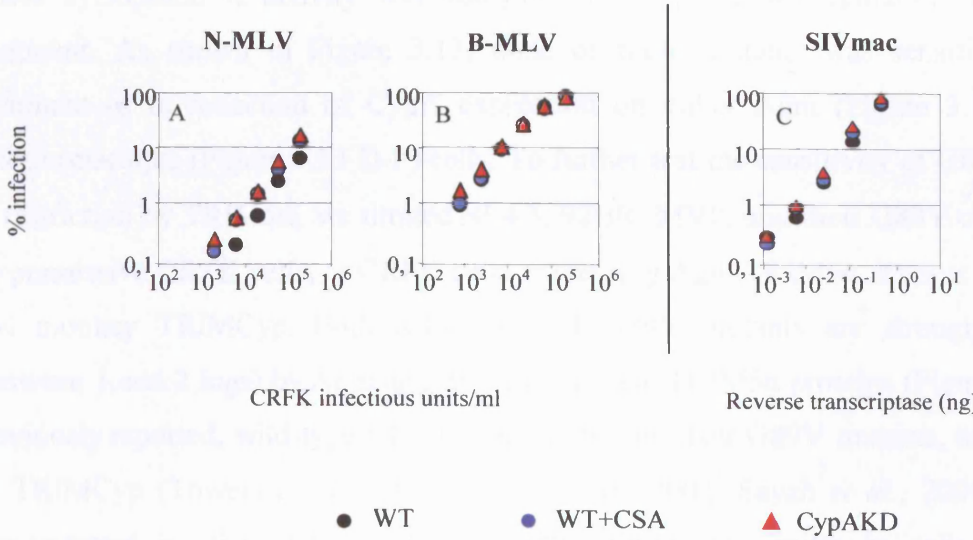


Figure 3.12. Cyclophilin A is not required for restriction of MLV or SIVmac by TRIM5α. Threefold serial dilutions of N-MLV (*A*), B-MLV (*B*) and SIV_{mac} (*C*) encoding GFP were titrated onto Agm CV1 cells (●), Agm CV1 cells in the presence of 5 μM CSA (●), or Agm CV1 cells expressing shRNA to CypA (CypAKD, ▲). MLV input doses are shown as CRFK infectious units per millilitre and SIVmac input doses are shown as nanograms of reverse transcriptase as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

3.2.6 TRIM5α restricts HIV-1 independently of CypA binding to HIV-1 CA

The cyclophilin A domain of TRIMCyp serves to recruit this potent restriction factor to the HIV-1 capsid. In the presence of cyclosporine A, or when the cyclophilin A binding loop of the HIV-1 capsid is mutated, TRIMCyp is unable to recognise the HIV-1 capsid, and thus loses its ability to restrict infection of this virus. The ability of TRIMCyp to restrict HIV-1 completely depends on the ability of the virus to bind cyclophilin A (Nisole *et al.*, 2004; Sayah *et al.*, 2004b). In order to examine if CypA recruits TRIM5α to HIV-1 in Agm and Rhesus macaque cells, we examined the infectivity of HIV-1 mutants (CA G89V), which are unable to bind cyclophilin A in these cells.

NL4.3 G89V, 92BR G89V and MVP G89V mutant viruses were titrated onto unmodified Agm CV1 and Rhesus macaque FRhK cells, and on cells where endogenous level of TRIM5α was stably downregulated by TRIM5-directed shRNA (Figure 3.13). The results showed that despite being unable to interact with CypA, all G89V HIV-1 mutants are still sensitive to TRIM5α (between 3- to 10-fold titre reduction) on Agm and Rhesus macaque cells (Figure 3.13). To confirm that the G89V mutants are insensitive to cyclophilin A, we compared their titre on unmodified CV1 and FRhK cells, and on cells where cyclophilin A activity was abrogated by CypA down-regulation, or by CSA treatment. As shown in Figure 3.13, none of these mutants was sensitive to CSA treatment or to reduction of CypA expression on either Agm (Figure 3.13 A-C) or Rhesus macaque (Figure 3.13 D-F) cells. To further test the sensitivity of G89V mutants to restriction by TRIM5α, we titrated NL4.3, 92BR, MVP, and their G89V counterparts, on permissive CRFK cells, or CRFK cells expressing Agm TRIM5α, Rhesus TRIM5α or owl monkey TRIMCyp. Both wild-type and G89V mutants are strongly restricted (between 1 and 2 logs) by Agm and Rhesus macaque TRIM5α proteins (Figure 3.14). As previously reported, wild-type HIV-1 viruses, but not their G89V mutants, are restricted by TRIMCyp (Towers *et al.*, 2003; Nisole *et al.*, 2004; Sayah *et al.*, 2004b). This is demonstrated by the fact that HIV-1 G89V titres are similar in cells expressing TRIMCyp and in unmodified permissive CRFK cells (Figure 3.14 D-F). These data demonstrate a clear difference in the role of CypA in restriction by TRIM5α and TRIMCyp. Whilst cyclophilin A is required for maximal restriction of HIV-1 by TRIM5α, TRIM5α contrasts TRIMCyp by being capable of restricting HIV-1 mutants in the absence of cyclophilin A activity.

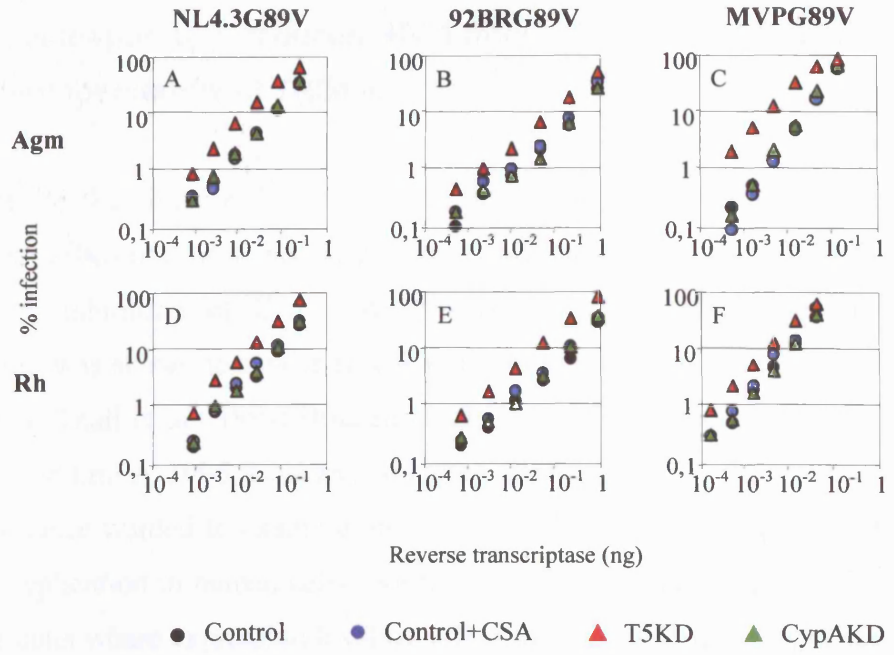


Figure 3.13. TRIM5α restricts HIV-1 independently of CypA binding to HIV-1 CA. HIV-1 NL4.3 G89V, 92BRG89V or MVP G89V were titrated onto Agm CV1 cells (*A to C*) and Rhesus FRhK4 (*D to F*) cells that were either untreated (●), treated with 5μM CSA (●), expressing reduced levels of TRIM5α protein (▲), or expressing reduced levels of CypA protein (▲). Virus input doses are measured in nanograms of reverse transcriptase, as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

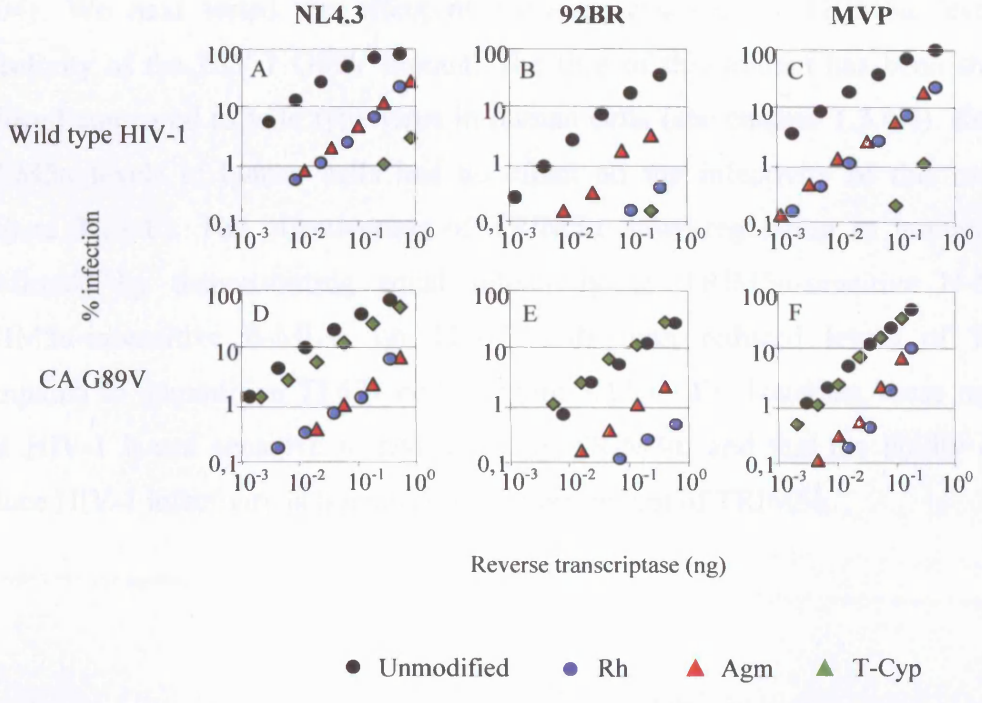


Figure 3.14. TRIM5α-mediated restriction of wild-type and CA G89V HIV-1 in feline CRFK cells. Wild-type HIV-1 NL4.3, 92BR or MVP5180 (*A to C*) and their mutant CA G89V counterparts (*D to F*) were titrated onto unmodified CRFK cells (●), or CRFK cells stably expressing Rhesus TRIM5α (●), Agm TRIM5α (▲), or owl monkey TRIMCyp (▲). Virus input doses are measured in nanograms of reverse transcriptase, as measured by ELISA. Results are representative of two independent experiments performed with two independent preparations of virus.

3.2.7 Cyclosporine A reduces HIV-1 infectivity in human cells independently of TRIM5α

Contrary to the situation in simian cells, in human cells cyclophilin A is required for maximal efficiency of a poorly-defined, post-entry, capsid-dependent step of HIV-1 infection. Inhibition of CA-CypA interactions with cyclosporine A, or by capsid mutation, was shown to render HIV-1 replication less efficient in these cells (Franke *et al.*, 1994; Thali *et al.*, 1994; Braaten *et al.*, 1996a, b, c; Dorfman and Gottlinger, 1996; Franke and Luban, 1996; Braaten and Luban, 2001).

We therefore wanted to examine the role of TRIM5α in the negative effect of CSA on HIV-1 replication in human cells. We titrated HIV-1 on unmodified TE671 cells and on TE671 cells where expression level of TRIM5α was stably downregulated by shRNA, in the presence or absence of 2.5 μM CSA (Figure 3.15). The results showed that reducing TRIM5α expression had no effect on the decrease of HIV-1 infectivity by CSA (Figure 3.15 D). Furthermore, TRIM5α has a little effect on HIV-1 replication in human cells (compare Figure 3.1 A and D), consistent with previous reports that HIV-1 is largely insensitive to restriction by human TRIM5α (Hatziioannou *et al.*, 2003; Stremlau *et al.*, 2004). We next tested the effect of reducing endogenous TRIM5α levels on the infectivity of the HIV-1 G89V mutant. The titre of this mutant has been shown to be reduced compared to wild type virus in human cells (see chapter 1.3.4.4). Reduction of TRIM5α levels in human cells had no effect on the infectivity of this mutant virus (Figure 3.15 E). The effectiveness of TRIM5α down-regulation in human cells was confirmed by demonstrating equal infectivity of TRIM5α-sensitive N-MLV, and TRIM5α-insensitive B-MLV, on TE671 cells with reduced levels of TRIM5, as compared to unmodified TE671 cells (Figure 3.15 C, F). Together, these results show that HIV-1 is not sensitive to restriction by TRIM5α, and that the ability of CSA to reduce HIV-1 infectivity in human cells is independent of TRIM5α.

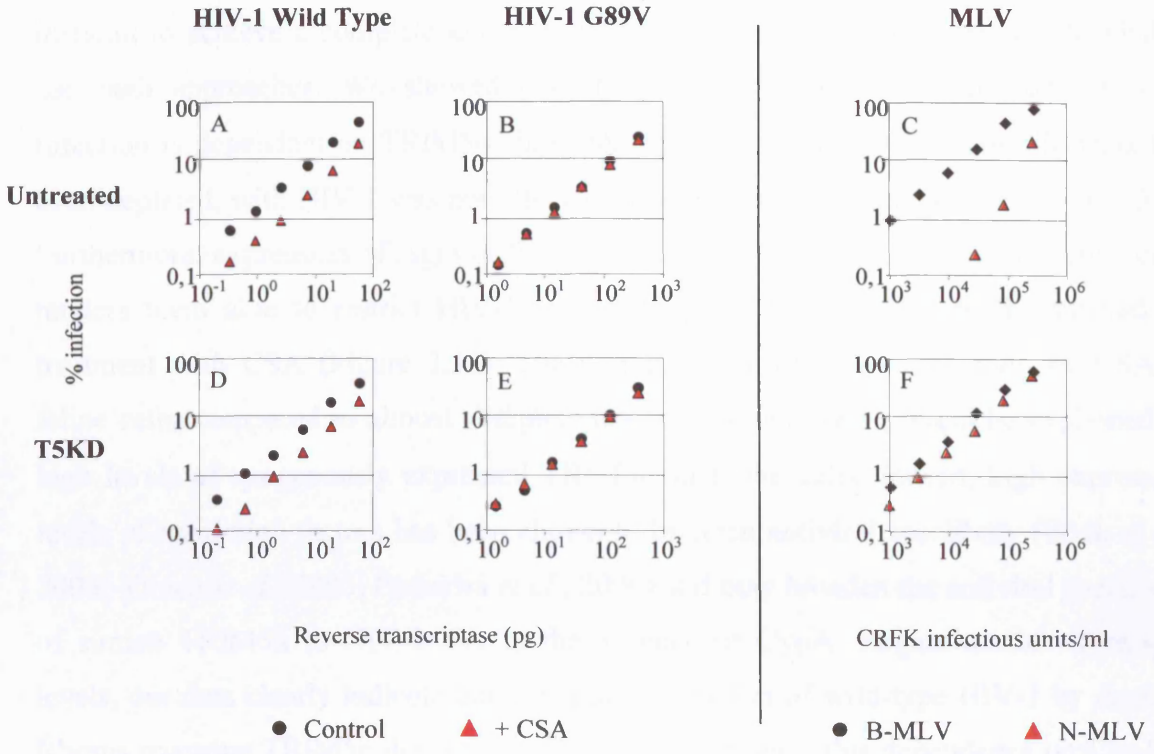


Figure 3.15. Cyclosporine A or the G89V mutation reduces HIV-1 infectivity in human cells independently of TRIM5α. Threefold serial dilutions of wild-type HIV-1 NL4.3 (*A and D*) or HIV-1 CA G89V (*B and E*) were titrated onto TE671 cells stably expressing TRIM5 shRNA (T5KD, *D and E*) or on to unmodified cells as a control (*A and B*) in the presence (▲) or absence (●) of 2.5 μM CSA. Threefold serial dilutions of N-MLV (▲) or B-MLV (●) were titrated onto TE671 cells expressing TRIM5 shRNA (*F*) or unmodified TE671 cells (*C*). HIV-1 input doses are measured in picograms of reverse transcriptase, as determined by ELISA. MLV input doses are shown as CRFK infectious units per millilitre. Results are representative of two independent experiments performed with two independent preparations of virus.

3.2.8 Discussion

We identified cyclophilin A as a host cell factor that is able to effect TRIM5α antiviral activity. We showed that the prolyl isomerase enzyme CypA has a negative effect on HIV-1 replication in Old World Monkey cells. Down-regulation of endogenous CypA levels or abrogation of CypA activity with cyclosporine A, significantly increased the efficiency of HIV-1 infection (Figure 3.8). Although working with cyclosporine A is easy, and time saving compared to the preparation of stable cyclophilin A knock-down cell lines, it also inhibits the enzymatic activity of immunophilins additional to cyclophilin A. On the other hand, the abundance of cyclophilin A within cells makes it difficult to achieve a complete knock-down of its expression. Therefore, we decided to use both approaches. We showed that the inhibitory effect of CypA on retroviral infection is dependent on TRIM5α. Infection of cells, where endogenous TRIM5α had been depleted, with HIV-1 was not affected by abrogation of CypA activity (Figure 3.9). Furthermore, expression of Agm or Rhesus macaque TRIM5α in permissive feline cells renders them able to restrict HIV-1, and this antiviral effect is partially relieved by treatment with CSA (Figure 3.11). The partial rescue of HIV-1 infection by CSA in feline cells, compared to almost complete rescue in simian cells, might be explained by high levels of exogenously expressed TRIM5α in feline cells. Indeed, high expression levels of restriction factors has been shown to broaden antiviral specificity (Bock *et al.*, 2000; Ylinen *et al.*, 2005; Passerini *et al.*, 2006) and may broaden the antiviral specificity of simian TRIM5α to HIV-1 CA in the absence of CypA. Regardless of expression levels, our data clearly indicate that maximal restriction of wild-type HIV-1 by Agm or Rhesus macaque TRIM5α depends on CypA. Furthermore, this dependence on CypA is only seen for TRIM5α-mediated restriction of HIV-1. This is consistent with the observation that, among the viruses tested in these experiments, HIV-1 is the only retrovirus known to package CypA into virions during viral assembly and to have an exposed proline residue on its CA subject to CypA-mediated peptidyl prolyl isomerisation (Braaten *et al.*, 1996b; Bosco *et al.*, 2002). Inactivation of CypA activity had no effect on the MLV or SIV_{mac} replication (Figure 3.12), and this not only supports the unique role of CypA in TRIM5α-mediated restriction of HIV-1, but also suggests that CypA acts on HIV-1 CA rather than on TRIM5α. Consistent with this notion is the inability to detect the direct interaction of CypA and TRIM5α by using either the yeast two-hybrid system, coimmunoprecipitation or fluorescence microscopy (Kumaran, N., unpublished data and Berthoux *et al.*, 2005b). We next investigated whether the sole

function of CypA in TRIM5α's antiviral activity is to bridge the restriction factor and viral capsid. This was shown to be the case for TRIMCyp where the cyclophilin A moiety of this potent restriction factor recruits the remaining RBCC motif to the virus. This leads to absolute sensitivity of restriction to either drugs or HIV-1 CA mutations that prevent CypA-CA interactions. However, as shown in Figure 3.13, this is not true for the mechanism of action of TRIM5α. Both, Agm and Rhesus macaque TRIM5α, whether expressed endogenously in simian cells (Figure 3.13) or exogenously in feline cells (Figure 3.14), are still able to significantly restrict the infection of the HIV-1 G89V CA mutant, which is unable to bind cyclophilin A. We therefore propose that it is not the recruitment of TRIM5α to the viral capsid via cyclophilin A that is responsible for restriction, but rather CypA-mediated isomerisation of a proline residue in the TRIM5α sensitivity-determinant of the HIV-1 capsid that sensitises it to restriction by Old World monkey TRIM5α (Figure 3.16). We propose that an interaction between CypA and incoming HIV-1 core, and subsequent prolyl isomerisation, leads to an alteration in the conformation of the capsid target for simian TRIM5α and that this leads to altered sensitivity to restriction.

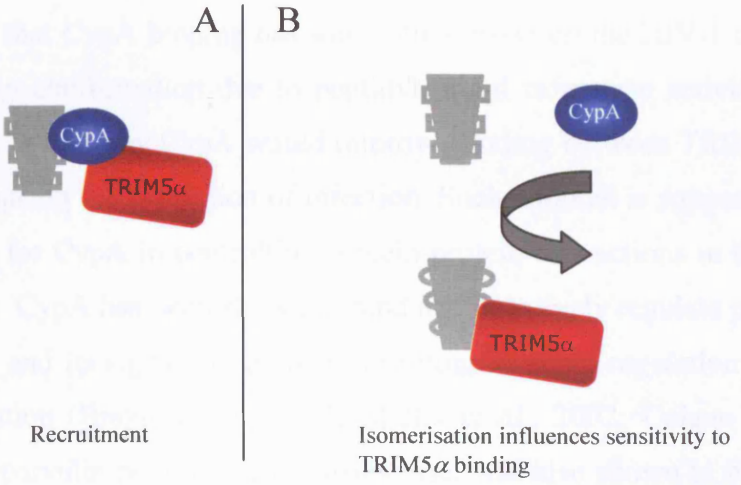


Figure 3.16. Two models of involvement of CypA in restriction. (*A*) binding of cyclophilin A (blue) to HIV-1 capsid (grey) recruits restriction factor TRIM5α (red). (*B*) cyclophilin A isomerises residues in HIV-1 capsid what renders HIV-1 capsid more susceptible to TRIM5α.

This model is supported by data which showed that the part of the SIV_{mac} CA, which corresponds to the cyclophilin A binding loop of HIV-1, contains the TRIM5α sensitivity-determinant, and mutations in this part of protein render SIV_{mac} insensitive to restriction by squirrel monkey and Rhesus macaque TRIM5α (Ylinen *et al.*, 2005). These

results suggest a role for this exposed part of the capsid in TRIM5α sensitivity, where an altered CA conformation might reasonably influence TRIM5α binding either directly or indirectly and subsequent restriction of infectivity. The possibility that CypA alters the HIV-1 CA's sensitivity to TRIM5α by P90 isomerisation is also supported by the observation that altering residues close to the CypA binding site in NL4.3(Ba-L) renders TRIM5α restriction of this virus independent of CypA activity, although the NL4.3(Ba-L) CA has been shown to bind CypA (Kootstra *et al.*, 2003; Chatterji *et al.*, 2005). These changes could either prevent CypA from isomerising P90, or the TRIM5α binding site could assume a conformation in which the isomerisation status of P90 does not impact on TRIM5α binding and restriction. Because the CypA binding virus NL4.3(Ba-L) is restricted in a CypA-independent way we discount a model in which the CypA-HIV-1 CA complex is a better target for TRIM5α restriction than HIV-1 CA alone. In the absence of CypA, Pro90 adopts kinetically trapped *cis* and *trans* conformations with nearly 86% of the HIV-1 CA Gly89-Pro90 covalent bonds in the *trans* conformation (Bosco *et al.*, 2002). Cyclophilin A catalyses the rapid isomerisation between these two states with the overall percentage of either isoform remaining unchanged. If TRIM5α recognises the *cis*-isomer more efficiently, then in the presence of CypA the 14% of the *cis* isoform would be renewed as it is taken out of the pool by binding to TRIM5α. It is, however, possible that CypA binding has some other effect on the HIV-1 capsid, but we believe a change in conformation due to peptidyl prolyl isomerase activity is the most likely explanation. In this way CypA would improve binding between TRIM5α and HIV-1 capsid thus enhancing the restriction of infection. Such a model is supported by studies considering a role for CypA in controlling protein-protein interactions in the case of the Itk tyrosine kinase. CypA has been shown to bind and negatively regulate protein-protein interactions of Itk and its signalling partners resulting in down-regulation of signalling, after TCR stimulation (Brazin *et al.*, 2002; Mallis *et al.*, 2002; Colgan *et al.*, 2004). Similarly, Pin1, a parvulin peptidyl-prolyl isomerase, was also shown to bind to and, by using its enzymatic activity, to alter the conformation of phosphoproteins involved in cell-cycle regulation. This leads to altered protein function and/or stability (reviewed in Yeh and Means, 2007).

Recent studies by Berthoux *et al.*, 2005b and Stremlau *et al.*, 2006b have also described an involvement of CypA in TRIM5α restriction of HIV-1. Although our data is consistent with the data of Stremlau *et al.* and also mostly support the work of Berthoux *et al.*, the difference with the latter work is that Berthoux *et al.* have proposed that the HIV-1 mutant CA G89V, which is unable to recruit CypA, is unrestricted in Agm and

Rhesus cells. Therefore, this study cannot differentiate between a model in which CypA recruits TRIM5α to the HIV-1 CA, as it does for TRIMCyp, and the model we propose in which CypA alters the conformation of the TRIM5α sensitivity-determinant and sensitivity to restriction. The difference in TRIM5α sensitivity of HIV-1 G89V between Berthoux's work on one side, and ours and Stremlau's on other side, remains unexplained.

In humans, the effect of cyclophilin A on HIV-1 infection is opposite to that seen in simian cells; abrogation of CypA activity renders HIV-1 less infectious. We, and others, have shown that this effect cannot be explained by alteration of TRIM5α activity and is thus TRIM5α-independent (Keckesova *et al.*, 2006; Sokolskaja *et al.*, 2006a; Stremlau *et al.*, 2006b). CSA treatment of human cells, where TRIM5α expression levels were stably or transiently downregulated by siRNA, had the same inhibitory effect on HIV-1 infection as in the wild type human cells (Figure 3.15). It was previously shown that CSA treatment enables wild-type HIV-1 to saturate restriction of N-MLV by TRIM5α in human cells (Towers *et al.*, 2003). This suggests that TRIM5α might be saturated by CSA-treated HIV-1, or it could also indicate saturation of a TRIM5α cofactor. The latter hypothesis is also supported by the isolation of a clone of human TE671 cells (17H1) that was selected for loss of N-MLV restriction. This clone was found to be permissive for HIV-1 in a CypA independent manner and the expression level of TRIM5α appears to be unchanged as compared to the wild type cells (Sayah *et al.*, 2004a, Luban, 2007). The existence of further CypA-sensitive antiviral factors might be supported by the observation that certain HIV-1 mutants, like HIV-1 CA A92E or G94D, appear to be restricted in a TRIM5α-independent way, in certain human cell lines (Sokolskaja *et al.*, 2004; Hatziioannou *et al.*, 2005). Interestingly, abrogation of CypA function increases their titre, as is the case with wild-type HIV-1 in simian cells, and infectivity of these mutant viruses is not rescued by high doses of mutant VLP (Sokolskaja *et al.*, 2004).

The notion of an involvement of immunophilins and/or peptidyl prolyl isomerisation in the modulation of protein interaction and in innate immunity is supported by numerous studies. The first hint of a role for CypA in immune function is supported by the use of CSA as a potent immunosuppressive agent. Immunosuppression results from the inhibition of calcineurin caused by the interaction between calcineurin and the CypA-CSA binary complex. Calcineurin inhibition prevents dephosphorylation of the transcription factor, nuclear factor of activated T-cells (NFAT), and its translocation to the nucleus, resulting in the inhibition of transcription of IL-2, IFN-γ, and other genes involved in T-cell activation. This was shown to occur only in the presence of CSA;

CypA on its own is not thought to regulate calcineurin. Interestingly, calcineurin is also inhibited by a complex between another immunophilin, FK506 binding protein (FKBP12), and the immunosuppressant FK506 (Liu *et al.*, 1991). The role of the FK506-binding proteins in TRIM5α-mediated HIV-1 restriction was also tested. Treatment of cells with FK506 or rapamycin, which are inhibitors of FK506-binding proteins, or downregulating the expression of several members of FK506 binding protein family by siRNA did not modulate HIV-1 infectivity (Stremlau *et al.*, 2006b; Keckesova, Z. data not shown). Thus, it seems that neither FK506-binding proteins nor calcineurin play a role in HIV-1 permissivity or in the TRIM5α restriction pathway.

Retroviruses are not the only viruses influenced by cyclophilin activity. Recent work showed that hepatitis C virus (HCV) is dependent on CypB activity for replication in human cells (Watashi *et al.*, 2005). Cyclophilin B was shown to directly interact with the HCV RNA-dependent RNA polymerase leading to a stimulated RNA binding and more efficient viral genome replication. Vaccinia virus also appears to depend on immunophilin activity for replication, where as much as 97% of virus yield is inhibited in the presence of CSA, although the mechanism of this antiviral effect remains unclear (Damaso and Moussatche, 1998). CypA is also found associated with vesicular stomatitis virus where it is thought to act as a chaperone for the extremely hydrophobic nucleocapsid protein (Cantin *et al.*, 2005). Apart from animals, an innate immune role for cyclophilins has also been found in plants. *Arabidopsis* cyclophilin indirectly activates specific innate immune resistance factor RPS2 by isomerising prolines on an incoming *Pseudomonas* protease AvrRpt2, thus causing its activation and leading to the elimination of RIN4; a negative regulator of RPS2 (Coaker *et al.*, 2005). This model is similar to that proposed here, in which CypA sensitis es HIV-1 to Old World monkey TRIM5α by isomerising proline residues on the incoming HIV-1 capsid. HIV-1's dependence on CypA activity in human cells might not contradict an innate immune role for cyclophilins. If cyclophilins contribute to innate immune function by isomerising prolines on incoming viral proteins, then in order to replicate in human cells HIV-1 may have evolved to tolerate CypA activity. This could lead to dependence on CypA if the prolines targeted are functionally important for viral replication. We note that a proline rich loop is found on the outer surface of all primate lentiviral capsids and this conservation supports an important role of this sequence in lentiviral replication.

The precise function of cyclophilins in cell biology remains unclear. A nonessential role, at least under laboratory growth conditions, is indicated by the fact that yeast knocked out for all eight cyclophilin genes remain viable (Dolinski *et al.*, 1997). On the other

hand, studies of CypA knockout mice clearly established its role *in vivo*. The CypA knock-out mice are viable but spontaneously develop an allergic disease reminiscent of interleukin-4 over-expression. A search for the mechanism led to the discovery that the PPIase active site of CypA binds and inactivates interleukin-2 tyrosine kinase thus suppressing the T helper 2 immune response (Colgan *et al.*, 2004). CypA was also shown to regulate the subcellular distribution of zinc-finger protein Zpr1 in *Saccharomyces cerevisiae* (Ansari *et al.*, 2002). Thus, it was hypothesised that CypA might also regulate TRIM5α localisation. However, the subcellular distribution of TRIM5α, as well as the formation of cytoplasmic bodies, was not altered by disruption of expression of CypA (Berthoux, L., unpublished data). Early studies indicated that HIV-1 and the closely related SIV_{cpz} from chimpanzees are the only retroviruses whose capsid protein binds CypA, and are dependent upon CypA for replication (Braaten *et al.*, 1996c). However, even within HIV-1, naturally occurring HIV-1 variants exist that do not depend on CypA for optimal infection of human cells (Braaten *et al.*, 1996c, Wiegers and Krausslich, 2002; Ikeda *et al.*, 2004; Chatterji *et al.*, 2005). Two additional lentiviruses have recently been shown to bind CypA and to be restricted by the TRIMCyp fusion protein, SIV_{Agm} from *Cercopithecus tantalus* and feline immunodeficiency virus (FIV) (Diaz-Griffero *et al.*, 2006b; Lin and Emerman, 2006; Zhang *et al.*, 2006). Moreover, the spreading infection of FIV is decreased by CSA in feline and human cells (Mortola *et al.*, 1998; Lin and Emerman, 2006). It will be interesting to test if CypA impacts on SIV_{Agm} or FIV replication in cells expressing unfused TRIM5α and if the effects of CSA depend on the presence of TRIM5α or related molecules. All these examples of cyclophilin activity suggest an important role for peptidyl prolyl isomerisation in immunity, and the further study of immunophilins may reveal important details of interactions between hosts and their pathogens.

3.3 Results 3

3.3.1 Introduction

Arsenic trioxide (As_2O_3) is commonly used to treat patients with acute promyelocytic leukaemia (APL). This disease is caused by an oncogenic promyelocytic leukaemia protein (PML)-retinoic acid receptor alpha (RARα) fusion protein resulting from chromosomal translocation. The fusion protein interferes with functions of both RARα and PML (de The *et al.*, 1991) and functions as a strong repressor of genes involved in myeloid differentiation, thus giving rise to promyelocytic leukaemia. Arsenic treatment has been shown to cause the degradation of the PML-RARα and wild type PML proteins (Lallemand-Breitenbach *et al.*, 2001) and to trigger cell death by apoptosis of APL cells. These events most likely contribute to the therapeutic effects of this drug (reviewed in Zhu *et al.*, 2002). Promyelocytic leukaemia protein (PML) belongs to the TRIM family of proteins and is required for the formation of discrete nuclear subdomains, known as PML nuclear bodies (Dyck *et al.*, 1994). Because of the common disruption of these bodies by infection with DNA viruses, one of the roles of these multiprotein complexes has been proposed to be antiviral (see chapter 1.3.4.1; Ahn *et al.*, 1998; Chee *et al.*, 2003; Rosa-Calatrava *et al.*, 2003). The biochemical pathways involved in the effect of arsenic on PML degradation have been partly unravelled. As_2O_3 treatment induces phosphorylation of PML through a mitogen-activated protein (MAP) kinase pathway, followed by increased PML sumoylation and translocation of PML to the nuclear matrix where it is subsequently degraded within the nuclear PML bodies (Zhu *et al.*, 1997; Lallemand-Breitenbach *et al.*, 2001; Hayakawa and Privalsky, 2004).

Some recent studies have focused on the effect of As_2O_3 and PML on retroviral infection. Turelli *et al.*, (2001) reported that As_2O_3 treatment of human cells enhanced HIV-1 infection in a PML-dependent way. In their study, HIV-1 infection caused a relocalisation of PML to the cytoplasm. They suggested that the addition of As_2O_3 might promote infection by sequestering PML in the nucleus, thus antagonising an HIV-1-antiviral activity associated with PML. They also found that the drug had no effect when added twelve hours post infection, suggesting that arsenic increases efficiency of an early post-entry step in the viral life cycle. Although subsequent studies confirmed the positive effect of As_2O_3 on HIV-1 infection they failed to confirm the role of PML in this effect (Bell *et al.*, 2001; Berthoux *et al.*, 2003). Berthoux *et al.*, (2003) extended these

observations by showing that arsenic trioxide also partially rescued N-MLV infection in human TE671 cells, thus counteracting Ref1 restriction.

Because Ref1 was shown to be encoded by TRIM5α, and both TRIM5α and PML are members of the tripartite motif containing family of proteins involved in cellular antiviral processes, it seems likely that the reported As_2O_3 effect in human cells might be explained by the effect of As_2O_3 on endogenous human TRIM5α. It is possible that the effects of As_2O_3 on these two proteins might also share some biochemical similarities. We therefore set up a series of experiments that would enable us to evaluate the role of TRIM5α in the arsenic-mediated effects on retroviral replication. Firstly, we needed to know if the stimulatory effect of arsenic trioxide is specific for N-MLV or if arsenic also affects the replication of another TRIM5α-restricted virus, equine infectious anaemia virus (EIAV). By stable downregulation of TRIM5α-expression in human TE671 cells we wanted to find out if As_2O_3 acts through the TRIM5α protein or if its enhancing effect on retroviral replication is due to interference with other TRIM5α-independent arsenic trioxide-influenced pathway. To access the role of degradation, translocation and/or sumoylation of TRIM5α in the arsenic trioxide-mediated effect on retroviral infection we used biochemistry, microscopy and mutagenesis studies.

3.3.2 Arsenic trioxide counteracts restriction in human but not Agm cells

If the reported stimulatory effect of As_2O_3 on N-MLV infection is due to its effect on TRIM5α we would expect the titre of other viruses inhibited by this restriction factor to be relieved by treatment with As_2O_3. In that case we would expect arsenic trioxide to rescue restriction of EIAV.

We therefore exposed human TE671 cells to VSV-G pseudotyped N-MLV, B-MLV and EIAV in the presence or absence of 4 μM As_2O_3. This dose of drug was shown to be optimal for the human TE671 cell line (Berthoux *et al.*, 2003). As_2O_3 was able to specifically rescue infectivity of EIAV GFP and N-MLV GFP by around 10-fold in human cells (Figure 3.17). The titre of unrestricted B-MLV remained unchanged (Figure 3.17). In contrast, treatment of the African green monkey CV1 cell line (*Cercopithecus aethiops tantalus*) with doses of As_2O_3 as high as 10μM did not increase N-MLV titre; it even had a slight inhibitory effect (Figure 3.18 A). B-MLV was included as a control (Figure 3.18 A). To exclude the possibility that the drug concentration was not high enough we infected CV1 cells with a fixed dose (multiplicity of infection of 0.01) of N-MLV and B-MLV in the presence of increasing doses of As_2O_3. The result showed, that

3.3.3 TRIM5α is required for the stimulatory effect of As_2O_3 on retroviral infection

Next we sought to test whether TRIM5α is required for the effect of arsenic trioxide on retroviral infectivity.

We therefore generated a TE671 cell line with stably reduced TRIM5 expression. Expression levels of TRIM5 were downregulated by stable introduction of a short hairpin RNA directed against TRIM5 into TE671 cells as described in *Materials and Methods* and we called these cells TEdT5. We then titrated N-MLV, B-MLV and EIAV vectors encoding GFP on TE671 and TEdT5 cells in the presence or absence of 4μM arsenic trioxide (Figure 3.19). The titre of N-MLV in TEdT5 cells was enhanced to the level of B-MLV, indicating efficient TRIM5 knockdown (compare Figure 3.19 B and D). While treatment of wild type TE671 cells with arsenic resulted in a 10-fold increase in N-MLV and EIAV infectivity (Figure 3.19 A, C) it had no effect on the titre of these viruses in the TRIM5- knockdown TEdT5 cells (Figure 3.19 D, F), thus demonstrating that As_2O_3 acts on TRIM5α and counteracts its antiviral activity. Unrestricted B-MLV was included as a control (Figure 3.19 B, E).

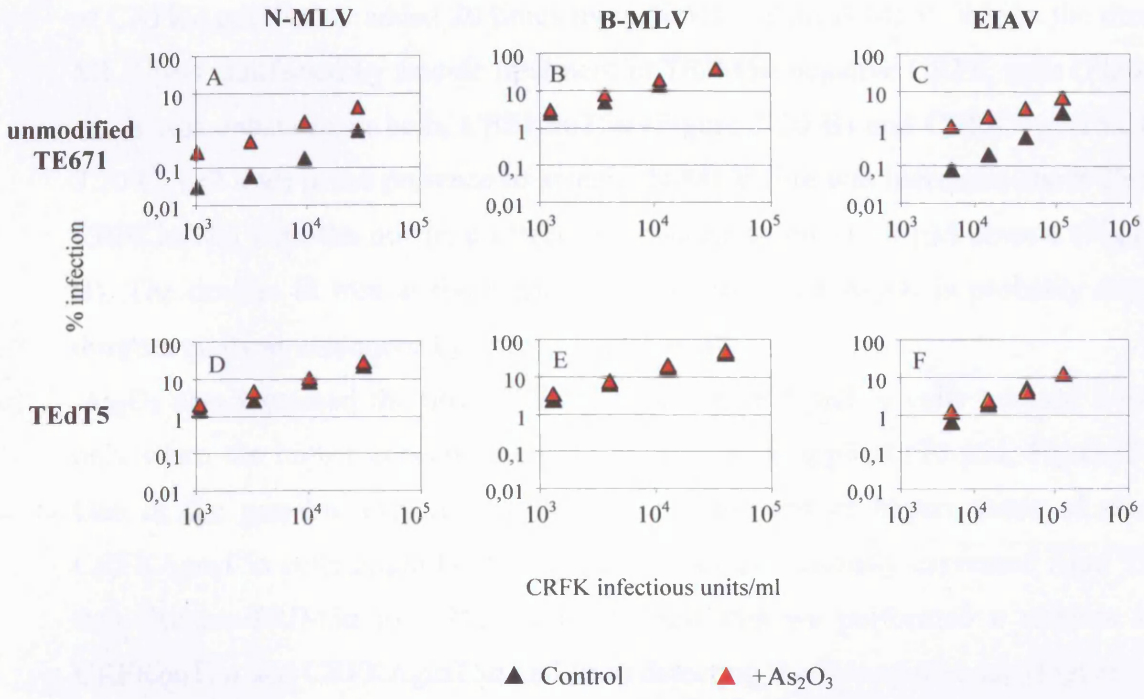


Figure 3.19. TRIM5α is required for the stimulatory effect of As_2O_3 on retroviral infection in human cells. Human TE671 cells and TE671 cells where TRIM5 expression was downregulated (TEdT5) were infected with titrations of N-MLV (*A, D*), B-MLV (*B, E*) and EIAV (*C, F*) virus vectors in the presence (▲) or absence (▲) of 4 μM arsenic trioxide. Virus input doses were measured in CRFK infectious units. Results are representative of two independent experiments performed with two independent preparations of virus.

3.3.4 Effect of As_2O_3 on retroviral restriction in cat cells expressing human and African green monkey TRIM5α proteins

The fact, that As_2O_3 was able to rescue infection in human TE671 cells, but not in Agm CV1 cells implied that the drug might suppress the restriction activity of some TRIM5α orthologues but not others. In order to find out if this differential effect of As_2O_3 is due to differences between the human and Agm TRIM5α alleles, or to differences between the human and Agm cells, we compared the effect of arsenic trioxide on the titre of N-MLV GFP in cat CRFK cells stably expressing human or Agm TRIM5α genes. Both, human and Agm TRIM5α cDNA, were fused to a haemagglutinin (HA) epitope tag at their C-terminus. We used infection with unrestricted B-MLV GFP and untransduced cells as controls.

Unmodified CRFK cells, and CRFK cells stably expressing human (CRFKhuT5α) or Agm (CRFKAgmT5α) TRIM5α genes were infected with N-MLV and B-MLV at a fixed multiplicity of infection (MOI of 0.01) in the presence of varying drug concentrations (Figure 3.20). In order to achieve similar multiplicity of infection we used more restricted N-MLV than unrestricted B-MLV in infectivity assays on restrictive cells. In the case of CRFKhuT5α we added 100 times more N-MLV than B-MLV and in the case of CRFKAgmT5α we added 20 times more N-MLV than B-MLV. Whilst the titre of N-MLV was unaffected by arsenic treatment in TRIM5α negative CRFK cells (Figure 3.20 A), it was enhanced in both, CRFKhuT5α (Figure 3.20 B) and CRFKAgmT5α (Figure 3.20 C) cell lines in the presence of arsenic. N-MLV titre was increased about 25-fold on CRFKhuT5α with the maximal effect at a concentration of 10 μM arsenic (Figure 3.20 B). The decline in titre at the highest concentrations of As_2O_3 is probably due to the drug's toxicity as evidenced by extensive cell death.

As_2O_3 also increased the titre of N-MLV on CRFKAgmT5α cells (around 5-fold) but only when the higher concentrations of arsenic were applied (20 μM, Figure 3.20 C). One of the possible explanations for the requirement of higher doses of arsenic in CRFKAgmT5α cells might be the higher level of exogenously expressed Agm TRIM5α than human TRIM5α in CRFK cells. To test this we performed a western blot on CRFKhuT5α and CRFKAgmT5α cell lines detecting the HA-epitope tag (Figure 3.20 D). Unmodified CRFK cells were included as a control for the specificity of the antibody against the haemagglutinin tag. Samples were equalized for protein levels using a Bradford assay. Even though both TRIM5α proteins are expressed from the same expression vector, the expression level of Agm TRIM5α was much higher than that of

human TRIM5α in 3 independent clones of CRFK cells. This would provide an explanation for the difference in arsenic sensitivity between these two cell lines. Interestingly, the toxic effects of arsenic trioxide were much less evident in CRFKAgmT5α cells than in CRFK cells expressing human TRIM5α. The reason for this is unclear but may suggest a protective effect of TRIM5α on the cells.

These results not only support the role of TRIM5α in the arsenic-mediated effect on retroviral infection, but also showed that the differential effect of As_2O_3 between TE671 and CV1 cells and between CV1 and CRFKAgmT5α cells is probably due to the intracellular environment and not due to intrinsic differences between the two TRIM5α alleles.

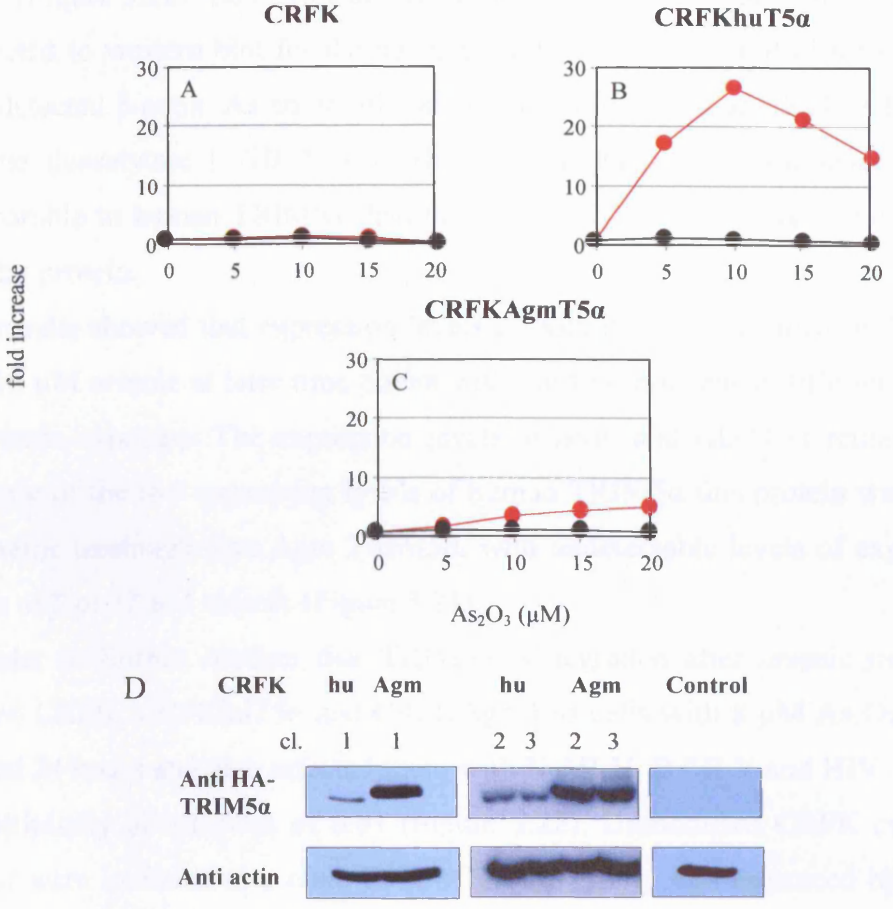


Figure 3.20. Retroviral infection of feline cells, expressing human and Agm TRIM5α proteins, is enhanced by As_2O_3 treatment. Unmodified CRFK (*A*) cells and CRFK cells stably expressing human (*B*) or Agm (*C*) TRIM5α protein were infected with N-MLV (•) and B-MLV (•) virus vectors at a multiplicity of infection of 0.01 in the presence or absence of increasing concentrations of arsenic trioxide (5, 10, 15, 20 μM). The fold increase in MLV infectivity was plotted. Results are representative of three independent experiments performed with three independent preparations of virus. (*D*) Western blot of extracts of unmodified CRFK (control) cells and CRFK cells stably expressing HA-tagged human (CRFKhu) and Agm (CRFKAgm). Samples were equalized by Bradford essay and probed with anti-HA antibody. α-Actin blots are shown as a control for equal loading. Three independent clones of CRFKhu and three independent clones of CRFKAgm were tested. The results shown in B, C were performed using CRFKhu clone 1 and CRFKAgm clone 1.

3.3.5 Degradation of human and African green monkey TRIM5α after arsenic trioxide treatment

The effect of arsenic on TRIM5α activity might be mediated through its deactivation either via translocation and/or degradation. Both of these are true for the PML (TRIM19) protein which, upon arsenic treatment, is translocated from the nucleoplasm to the nuclear matrix-associated PML bodies and subsequently degraded. To address the role of degradation of TRIM5α after arsenic treatment we treated CRFKhuT5α (Figure 3.21 A) and CRFKAgmT5α (Figure 3.21 B) cells, where the TRIM5α protein was appended with a C-terminal HA epitope, with increasing concentrations of As_2O_3 for 2, 6, 12, and 24 hours (Figure 3.21). Cell extracts were equalised for total protein by Bradford essay and subjected to western blot for the haemagglutinin tag. As a control for equal loading we also detected β-actin. As an additional control, in the case of CRFKhuT5α, we detected histone deacetylase-1 (HDAC-1). This protein has expression levels that are more comparable to human TRIM5α than those of actin which is a very stable and abundant cellular protein.

The results showed that expression levels of both proteins declined in the presence of 8 and 12 μM arsenic at later time points with the first noticeable difference after 12 hours of arsenic exposure. The expression levels of actin and HDAC-1 remained unchanged. Because of the low expression levels of human TRIM5α this protein was more sensitive to arsenic treatment than Agm TRIM5α, with undetectable levels of expression after 24 hours of 8 or 12 μM arsenic (Figure 3.21).

In order to further confirm that TRIM5α is degraded after arsenic treatment we pre-treated CRFK, CRFKhuT5α and CRFKAgmT5α cells with 8 μM As_2O_3 for 0.5, 1, 2, 6, 12 and 24 hours and then infected them with N-MLV, B-MLV and HIV-1 viral vectors at a multiplicity of infection of 0.01 (Figure 3.22). Unmodified CRFK cells and B-MLV vector were included as a control. N-MLV infectivity was enhanced by around 20-fold when the CRFKhuT5α cell line was exposed to As_2O_3 for 12 hours (Figure 3.22 B) and around 9-fold when the CRFKAgmT5α cell line was subjected to the same conditions (Figure 3.22 C). HIV-1 titre increased by around 5-fold on both TRIM5α-expressing cell lines after 12 hours of arsenic pre-treatment (Figure 3.22 B, C). B-MLV titre remained unchanged in all cell lines and under all conditions tested. Similarly, titres of N-MLV, B-MLV or HIV-1 vectors on wild type CRFK cells were unchanged (Figure 3.22 A).

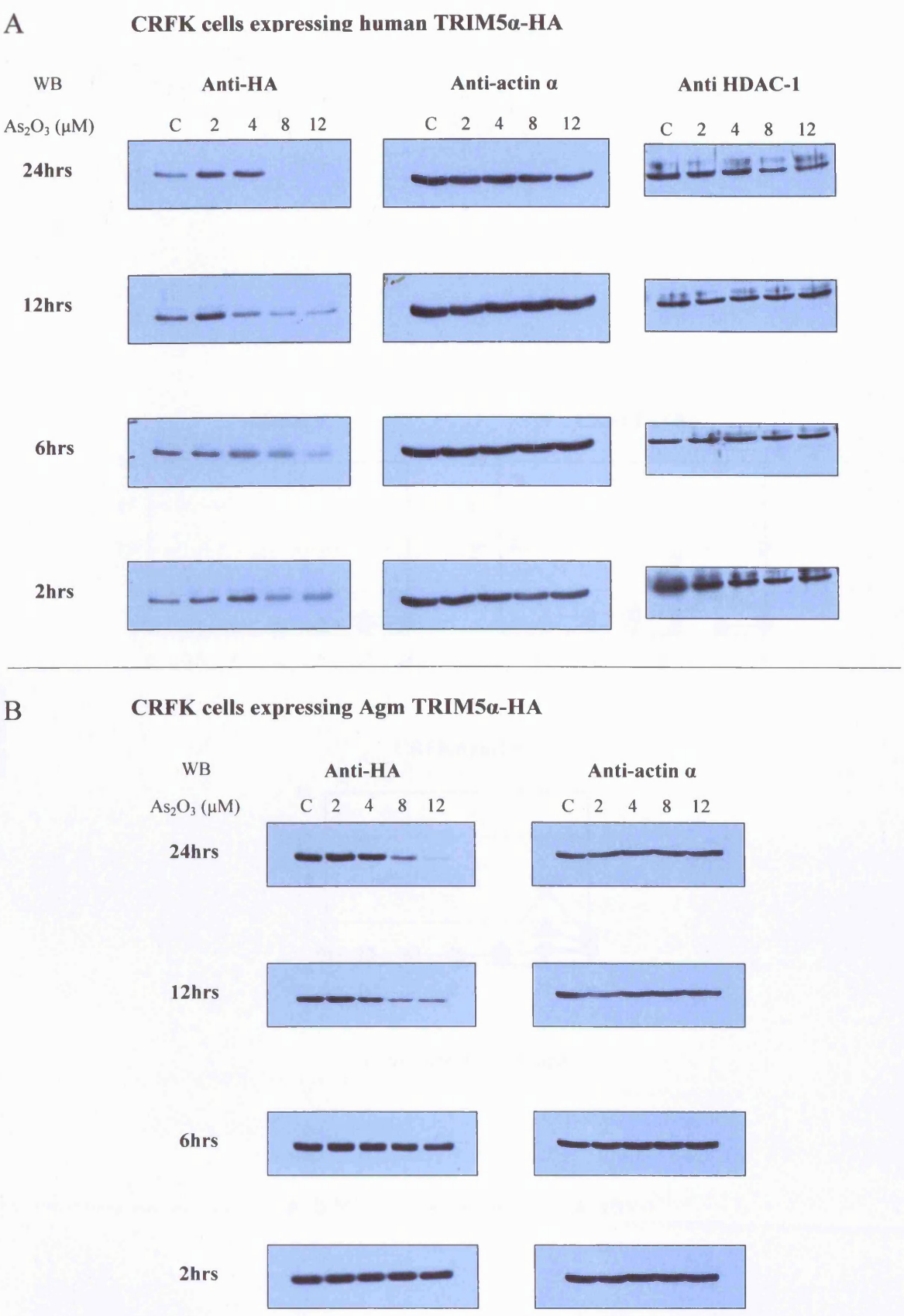


Figure 3.21. Degradation of human and African green monkey TRIM5α proteins by arsenic trioxide. CRFK cells expressing HA-tagged TRIM5α proteins from human (A) or Agm (B) were subjected to 2, 4, 8, or 12 μM As_2O_3 for 2, 6, 12 and 24 hours. Cell extracts were equalized for total protein by Bradford assay and 100ng of protein was loaded. Western blots (WB) were probed for HA-tag (*A, B*), α-actin (*A, B*) or histone deacetylase-1 (*A*). Results are representative of two independent experiments performed with two independent cell clones.

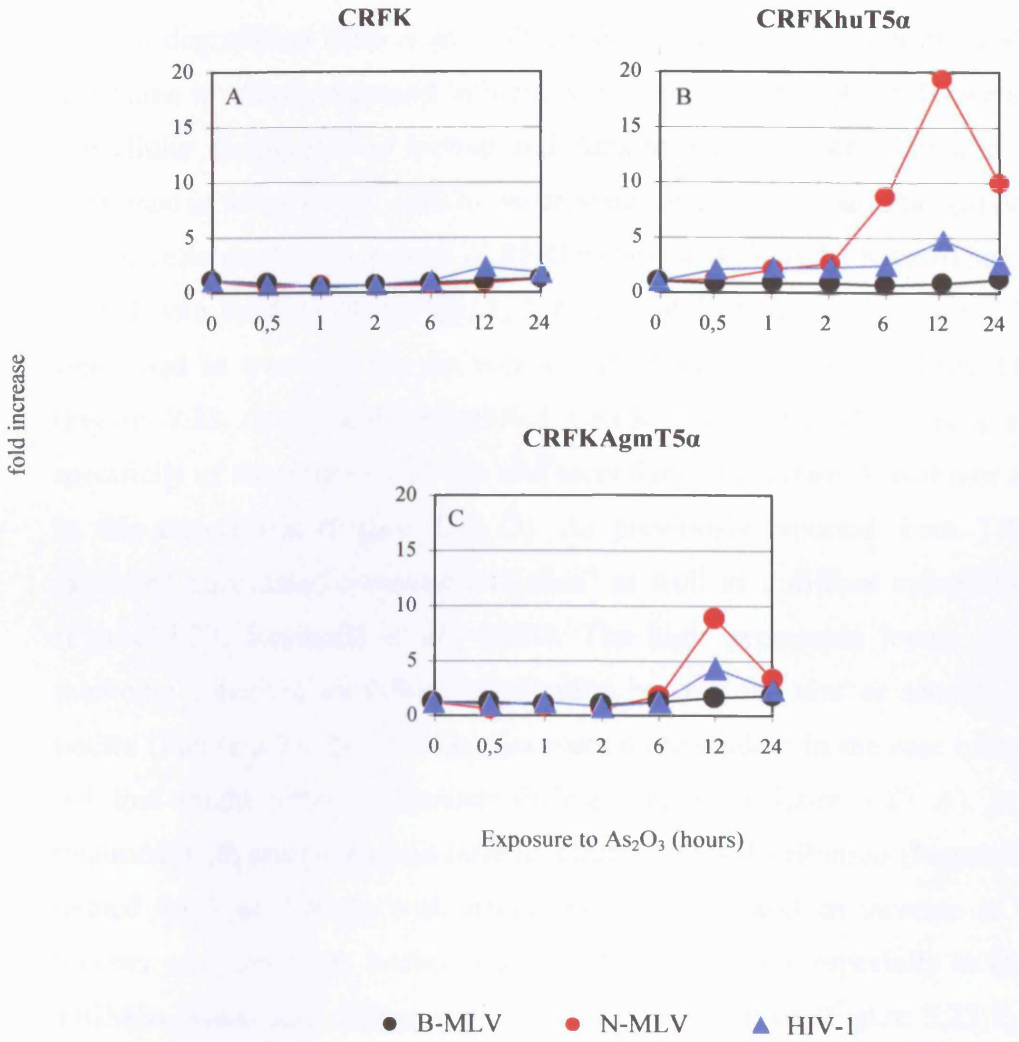


Figure 3.22. Pre-treatment of CRFKhuT5α and CRFKAgmT5α cells with arsenic trioxide. Unmodified CRFK (*A*) cells or CRFK cells stably expressing human (*B*) or Agm (*C*) TRIM5α protein were subjected to 8 μM arsenic treatment for 0.5, 1, 2, 6, 12 and 24 hours and then infected with N-MLV (●), B-MLV (●) and HIV-1 (▲) viral vectors at a MOI of 0.01. The fold increase in retroviral infectivity was plotted on the *y* axis. Results are representative of two independent experiments performed with two independent preparations of virus.

3.3.6 Examination of TRIM5α localisation in the presence of arsenic trioxide

Most of the experiments that showed the stimulatory effect of arsenic trioxide on retroviral infection, were performed with the drug added simultaneously with the virus (Bethoux *et al.*, 2003; Keckesova *et al.*, 2004; Sebastian *et al.*, 2006) and arsenic was shown to exert its effect on viral replication early in the viral life cycle, before reverse transcription (Berthoux *et al.*, 2003). Therefore, it seems improbable that the degradation of TRIM5α by arsenic would be responsible for arsenic's fast effect on retroviral replication. In the case of the PML protein, arsenic was shown to cause its translocation prior to degradation (Zhu *et al.*, 1997; Lallemand-Breitenbach *et al.*, 2001). In order to determine if arsenic treatment influences the localisation of TRIM5α we investigated the subcellular distribution of human and African green monkey TRIM5α proteins stably expressed in feline CRFK cells in the presence or absence of arsenic trioxide.

CRFK cells expressing human (CRFKhuT5α) or Agm (CRFKAgmT5α) TRIM5α were treated with 8μM As_2O_3 for 0.5, 1, 2, 6, 12 and 24 hours (Figure 3.23). Untreated cells were used as a control for the normal subcellular distribution of the TRIM5α protein (Figure 3.23 A, H) and unmodified CRFK cells were added as a control for the specificity of the primary anti-HA and secondary anti-mouse AlexaFluor antibodies used in this experiment (Figure 3.23 O). As previously reported, both TRIM5α proteins exhibited punctuate "cytoplasmic bodies" as well as a diffuse cytoplasmic distribution (Figure 3.23; Reymond *et al.*, 2001). The high expression levels of Agm TRIM5α markedly enhanced its diffuse distribution but not the size or number of cytoplasmic bodies (Figure 3.23 H). These bodies were more evident in the case of human TRIM5α, but this might reflect a dimmer diffuse staining (Figure 3.23 A). A thirty minute treatment with arsenic did not have an effect on this distribution (Figure 3.23 B, I). Cells treated for 1 or 2 hours with arsenic however, showed an increase in brightness and number of cytoplasmic bodies (Figure 3.23 C, D, J, K), especially in the case of Agm TRIM5α where less diffuse staining was also observed (Figure 3.23 J, K). A 6 hour treatment had the most pronounced effect on TRIM5α localisation with human TRIM5α being localised within large, bright structures (Figure 3.23 E). Most cells contained only one or two of these bodies. The brightness and size of the Agm TRIM5α bodies also increased but not to such an extent as in the case of the human TRIM5α (Figure 3.23 L). At 12 and 24 hours of treatment with arsenic trioxide the cytoplasmic aggregates of human TRIM5α protein were lost and less diffuse staining was seen (Figure 3.23 F, G). After 12 hours of As_2O_3 treatment Agm TRIM5α was still localised in a few cytoplasmic

bodies but the strong diffuse staining was lost (Figure 3.23 M). At 24 hours the cytoplasmic bodies were also lost (Figure 3.23 N) and cell death was evident for both, CRFKhuT5α and CRFKAgmT5α, cell lines (Figure 3.23 G, N).

In summary, within the first 2 hours of treatment, As_2O_3 had only subtle effects on the subcellular localisation of TRIM5α proteins expressed in cat cells. The most noticeable effects in the cytoplasmic distribution were seen at, and after 6 hours of arsenic treatment when both human and Agm TRIM5α proteins were localised within large, bright bodies which later disappeared. These structures are likely to represent the sites where TRIM5α proteins are degraded.

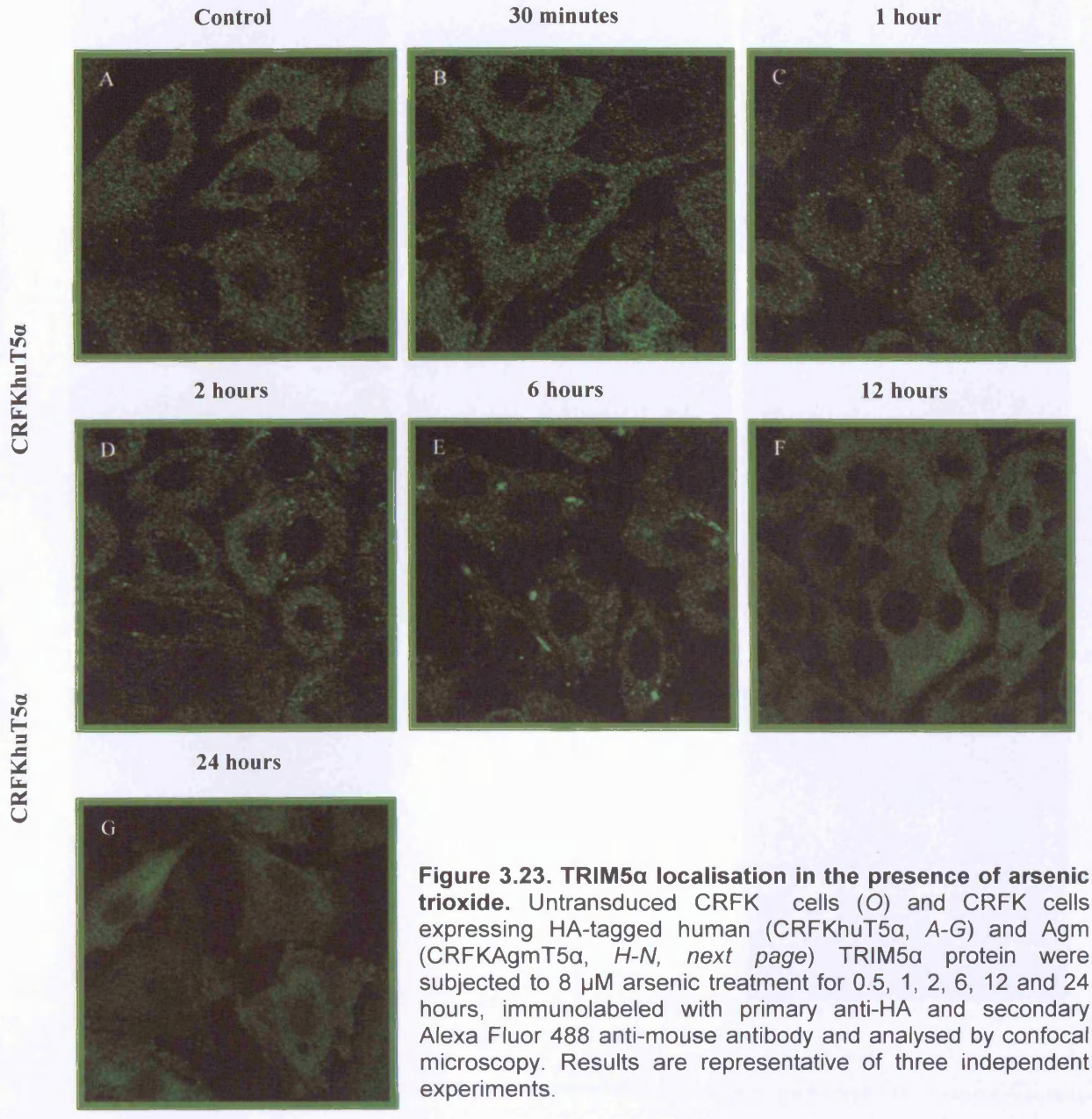
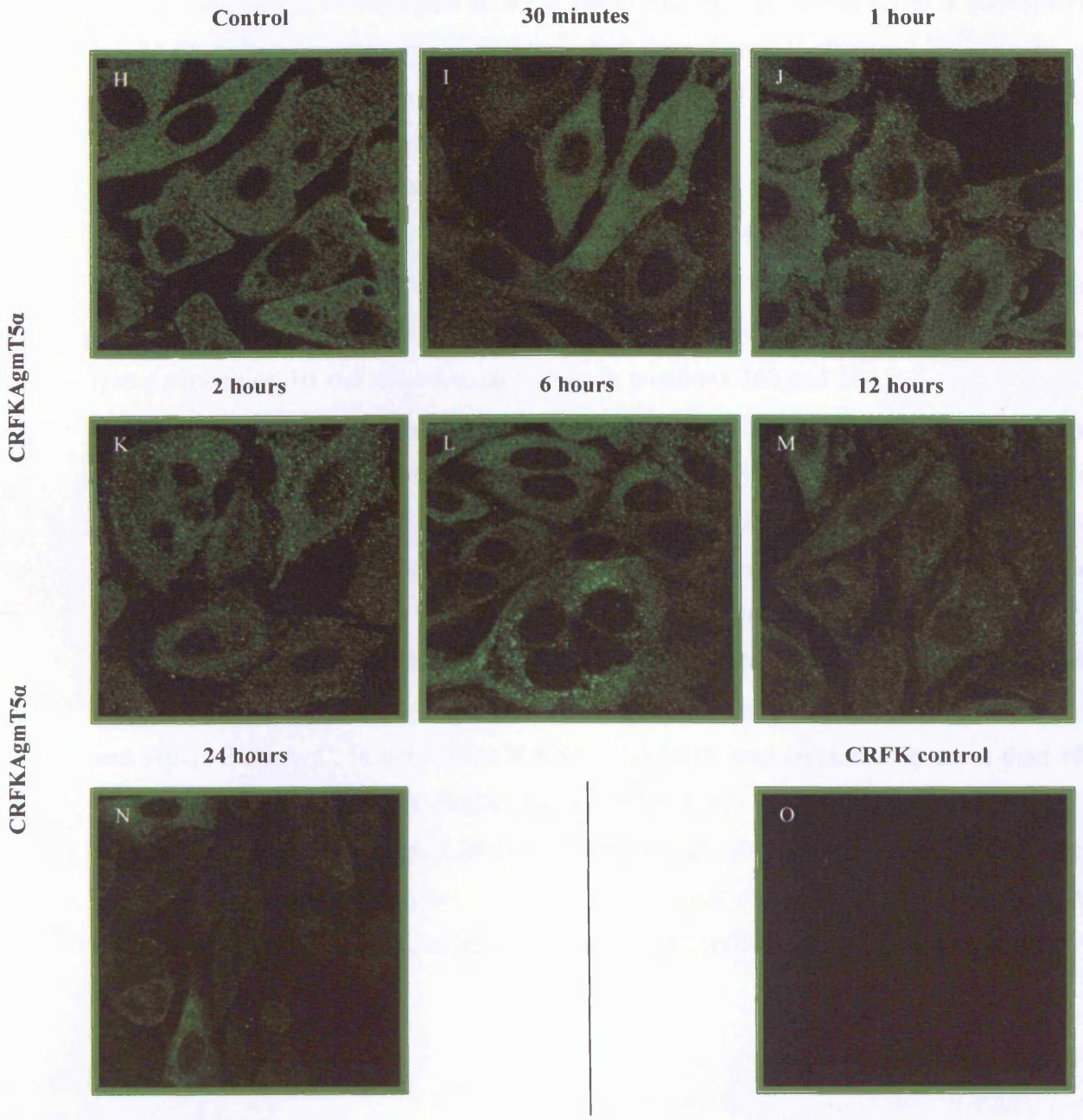


Figure 3.23. TRIM5α localisation in the presence of arsenic trioxide. Untransduced CRFK cells (*O*) and CRFK cells expressing HA-tagged human (CRFKhuT5α, *A-G*) and Agm (CRFKAgmT5α, *H-N, next page*) TRIM5α protein were subjected to 8 μM arsenic treatment for 0.5, 1, 2, 6, 12 and 24 hours, immunolabeled with primary anti-HA and secondary Alexa Fluor 488 anti-mouse antibody and analysed by confocal microscopy. Results are representative of three independent experiments.


Control
30 minutes
1 hour
H
I
J
CRFKAgmT5a
2 hours
6 hours
12 hours
K
L
M
CRFKAgmT5a
24 hours
CRFK control
N
O

3.3.7 Mutagenesis of putative TRIM5α sumoylation sites

In the case of PML, its degradation by arsenic treatment is preceded by its phosphorylation and sumoylation (Lallemand-Breitenbach *et al.*, 2001; Hayakawa and Privalsky, 2004). Sumoylation is a post-translational modification. The SUMO (small ubiquitin-like modifier) protein is covalently attached to other proteins on lysine residues. Sumoylation does not usually trigger degradation but rather modulates protein-protein interactions. Sumoylation at single site of the PML protein at K160 is responsible for As_2O_3-induced proteasome recruitment and degradation (Lallemand-Breitenbach *et al.*, 2001). Is it therefore possible that degradation of TRIM5α, and thus its arsenic-responsiveness, is also mediated via sumoylation?

Sumoylation occurs on a Ψ-K-X-D/E consensus where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, X is any amino-acid and D and E are acidic residues. A SUMOplot prediction (www.abgent.com/doc/sumoplot) revealed two motifs within human and Agm TRIM5α proteins with a high probability of sumoylation. They are the lysine at position 10 and two adjacent lysines in positions 263 and 264.

In order to test if these two putative sumoylation sites are involved in the As_2O_3-mediated effect on retroviral replication we mutated lysines within these motifs to arginines. HuK10R and huKK263,264RR TRIM5α proteins were then stably expressed in feline CRFK cells and analysed for their ability to restrict N-MLV and for their sensitivity to As_2O_3 (Figure 3.24 A, C). B-MLV was included as a control (Figure 3.24 B, D). Both of the mutant TRIM5α proteins restricted N-MLV with a similar magnitude to wild type human TRIM5α, by around two orders of magnitude; compare Figure 3.2 and Figure 3.24 A, C. In both cases N-MLV infectivity was enhanced by more than 10-fold by treatment with 8μM As_2O_3 (Figure 3.24 A, C). B-MLV infectivity remained unchanged in all cases (Figure 3.24 B, D). These results showed that mutating the lysines within these putative sumoylation sites had no effect on TRIM5α function or on its sensitivity to arsenic trioxide, at least not when these motifs were mutated individually.

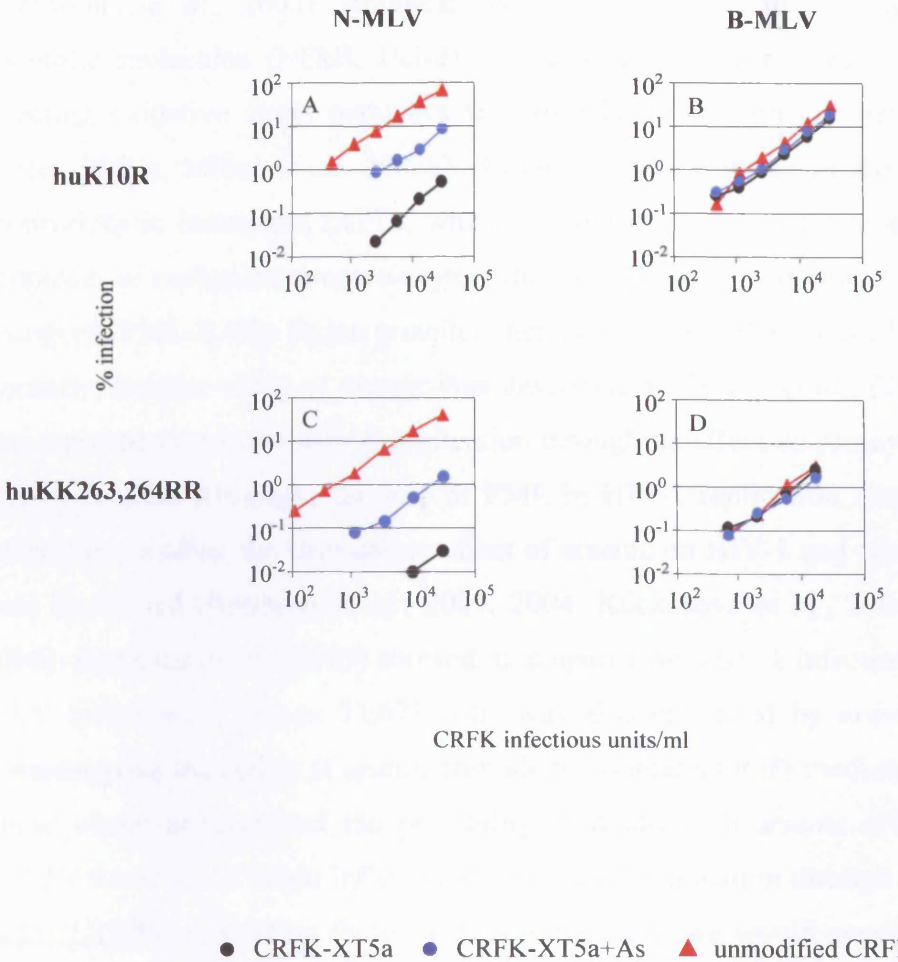


Figure 3.24. Mutagenesis of putative TRIM5α sumoylation sites does not impact on TRIM5α-mediated restriction or sensitivity to As_2O_3. Unmodified CRFK cells (triangles) and CRFK cells stably expressing human TRIM5α (circles) mutated either in K10 (huK10R, *A, B*) or in KK263,264 (huKK263,264RR, *C, D*) were infected with titrations of N-MLV (*A, C*) and B-MLV (*B, D*) virus vectors in the presence (●) or absence (●) of 8 μM arsenic trioxide. Virus input doses were measured in CRFK infectious units per milliliter. Results are representative of two independent experiments performed with two independent preparations of virus.

3.3.8 Discussion

In this study, we have examined the effect of arsenic trioxide on TRIM5α-mediated retroviral restriction. Arsenicals have accompanied the human population for more than two thousand years and have been used either as a medicine for treatment of various diseases, or as a poison. Nowadays, many of the arsenic-induced biochemical pathways have been partly elucidated. Arsenic was shown to affect numerous cellular signalling molecules and transduction pathways and to cause many alterations in cellular biochemistry. The action of arsenic may result in activation of differentiation, senescence, cell growth arrest and apoptosis (Kaltreider *et al.*, 2001; Halicka *et al.*, 2002; Chelbi-alix *et al.*, 2003). Apoptosis is triggered by arsenic through inhibiting anti-apoptotic molecules (NFkB, Bcl-2), stimulating apoptotic ones (caspases, p53) and affecting oxidative stress pathways and mitochondrial pore permissivity (reviewed in Miller, 2002a; Miller *et al.*, 2002b). As_2O_3 has 87% efficacy in the treatment of acute promyelocytic leukaemia (APL), where it induces partial differentiation and promotes apoptosis of malignant promyelocytes, through the inactivation and degradation of the oncogenic PML-RARα fusion protein (Chen *et al.*, 1996; Shao *et al.*, 1998).

Recently, another effect of arsenic was described by Turelli *et al.*, (2001), where As_2O_3 was reported to enhance HIV-1 replication through its effect on promyelocytic leukaemia (PML) protein. Although, the role of PML in HIV-1 replication was not confirmed by subsequent studies, the stimulatory effect of arsenic on HIV-1 and other retroviruses was later confirmed (Berthoux *et al.*, 2003, 2004; Keckesova *et al.*, 2004; Sebastian *et al.*, 2006). Berthoux *et al.*, (2003) showed, that apart from HIV-1 infection in HeLa cells, N-MLV infection in human TE671 cells was also enhanced by arsenic treatment, thus demonstrating the ability of arsenic trioxide to counteract Ref1 mediated restriction.

These observations raised the possibility, that although arsenic effects many diverse cellular functions, it might influence the retroviral replication through deregulation of the Ref1/ TRIM5α restriction factor. If this is the case, we would expect the titre of other Ref1 restricted viruses to be enhanced by arsenic treatment, while the titre of unrestricted viruses would remain unchanged. As Figure 3.17 shows this was indeed the case. The infection of human TE671 cells by equine infectious anaemia virus (EIAV), another Ref1 restricted virus, was enhanced in the presence of arsenic while the titre of unrestricted B-MLV was not affected. To further test if the effects of arsenic trioxide are dependent on the presence of TRIM5α, a factor that encodes Ref1 activity, we knocked-down the endogenous human TRIM5α protein in TE671 cells. Figure 3.19 shows, that retroviral

infection of cells, in which TRIM5α-expression levels had been downregulated, were unresponsive to arsenic treatment. This confirms that the effect of arsenic trioxide on retroviral infection is dependent on the presence of human TRIM5α.

In contrary to human cells, N-MLV infection of CV1 cells from African green monkey (Agm) was unaffected by arsenic treatment, even at high arsenic concentration (Figure 3.18; Berthoux *et al.*, 2003; Sebastian *et al.*, 2006), although they express a TRIM5α protein capable of restricting various viruses (Hatziioannou *et al.*, 2004b; Keckesova *et al.*, 2004; Yap *et al.*, 2004). This implied, that the drug might be active against some TRIM5α orthologues but not others. In order to resolve this, we stably expressed both, human and Agm TRIM5α proteins in feline CRFK cells and tested their responsiveness to arsenic. As Figure 3.20 shows, As_2O_3 increased the titre of N-MLV on both of the transduced CRFK cell lines, while it did not have an effect on unmodified CRFK cells. We reported previously (Keckesova *et al.*, 2004) that CRFK cells expressing Agm TRIM5α, as well as CV1 cells, did not respond to arsenic, but the concentrations of arsenic used were lower than 10μM. While the effect of arsenic on N-MLV replication in CRFKhuT5α cell line was very strong even at lower arsenic concentration (around 20-fold at 5μM arsenic concentration), N-MLV was enhanced by 5-fold at most on CRFKAgmT5α cells, even when the arsenic concentration was as high as 20μM. Subjecting these cells to western analysis revealed, that the differences in arsenic sensitivity on these cell lines can be attributed to much higher expression levels of Agm TRIM5α than those of human TRIM5α in all the clones tested. The difference in expression levels between CRFK cells expressing human and Agm TRIM5α proteins was also reported by Sebastian *et al.*, (2006), although in this study the TRIM5α was expressed using a different expression vector. Perhaps, high expression levels of human TRIM5α are toxic for the cell and only clones with low levels of human TRIM5α survive. In concordance, analysis of TE671 cells showed that endogenous TRIM5α protein is expressed at very low levels (Berthoux *et al.*, 2005a). It is possible that Agm TRIM5α is more stable with a slower turn over rate than that of human TRIM5α, the half-life of which was shown to be around 60 minutes (Diaz-Griffero *et al.*, 2006a). These results not only supported the role of TRIM5α in the arsenic-mediated effect on retroviral infection but also showed that the differential effect of As_2O_3 between TE671 and CV1 cells and between CV1 and CRFK cells expressing Agm TRIM5α might be due to the cell environment as was suggested by Sebastian *et al.*, 2006. On the other hand, it is possible that the unresponsiveness of CV1 cells to arsenic treatment might simply be explained by their high TRIM5α-expression levels or inefficient cellular drug uptake.

This is supported by the fact that no toxic effects of the drug were evident on CV1 cells, even at concentration as high as 20μM. Because of the unavailability of a specific anti-TRIM5α antibody the endogenous levels of TRIM5α protein in CV1 could not be accessed. CRFK cells expressing human TRIM5α protein exhibited extensive cell death at 20μM As_2O_3 while toxicity on CRFKAgmT5α cells was less evident with only a small percentage of cells being killed. Thus, the ability of As_2O_3 to enhance retroviral replication correlated well with the increase of toxic effects of the drug. Furthermore, the more TRIM5α protein was present in the cell the less toxic effects As_2O_3 exerted on these cell and the less it was able to enhance retroviral replication. The ability of arsenic to optimally stimulate retroviral replication at toxic concentrations was also observed by Berthoux *et al.*, 2003. They also showed, that it is not the general effect of apoptosis that caused the enhancement of viral infection. Titre of sensitive viruses remained unchanged on cells treated with apoptosis-inducing agents like camptothecin, cisplatin, and anti-Fas antibody. Attempts to lower the expression levels of Agm TRIM5α protein in CRFK cells were either unsuccessful or clones that were expressing amount of Agm TRIM5α comparable to human TRIM5α were impaired in their ability to restrict. Therefore, we still cannot rule out the possibility that Agm TRIM5α, because of some intrinsic differences between Agm and human TRIM5α orthologues, does not respond to arsenic as well as the human protein.

All the results presented so far showed, that As_2O_3 positively influences retroviral infection through its effect on the TRIM5α protein. This might happen through deregulation, inactivation, translocation or degradation of the TRIM5α protein. As_2O_3 was shown to cause the translocation and degradation of the TRIM5-related protein PML (Lallemand-Breitenbach *et al.*, 2001). Whether other members of the TRIM family are affected by arsenic trioxide remains untested. We have shown that both of the TRIM5α proteins were degraded in the presence of arsenic with the first noticeable decrease at 12 hours of arsenic treatment (Figure 3.21). This observation was also confirmed by enhancement of N-MLV infection in CRFKhuT5α and CRFKAgmT5α cells pre-treated with arsenic for 12 hours (Figure 3.22). Similarly, PML was shown to be degraded 8 to 24 hours after arsenic preexposure (Lallemand-Breitenbach *et al.*, 2001). It is possible therefore, that arsenic exerts this effect on more than just these two TRIM proteins. This might have therapeutic implications in the treatment of diseases caused by this large family of proteins.

Because arsenic was shown to act early in the viral infection cycle, we next examined the subcellular localisation of human and Agm TRIM5α proteins at various time points

(Figure 3.23). Arsenic had no strong effect on TRIM5α subcellular distribution at the earlier time points. Later, the number, size and brightness of cytoplasmic bodies slowly increased with the longer exposures of cells to arsenic. By 6 hours human TRIM5α expressed in CRFK cells was localised within a few bright cytoplasmic structures. A similar, although less obvious effect was observed in CRFKAgmT5α cells. The delay on the effects of arsenic on the Agm TRIM5α protein might be explained by the higher level of this protein within the cell. Diffuse cytoplasmic staining of both proteins, mainly Agm TRIM5α, is slowly decreasing in time with longer As_2O_3 exposure. By 12 and 24 hours the cytoplasmic bodies are lost and cell death becomes prevalent. These events most probably represent TRIM5α degradation. Previous studies implied, that cytoplasmic bodies are not important for TRIM5α activity (Perez-Caballero *et al.*, 2005a; Song *et al.*, 2005a). It was suggested that, they might simply represent sites where over-expressed TRIM5α protein is localised and later degraded (Diaz-Griffero *et al.*, 2006a; see chapter 1.3.4.5). This theory is consistent with the data presented here.

Although we showed, that the localisation of TRIM5α changes after 6 hours of arsenic treatment it is probably not responsible for the observed enhancement of retroviral replication. As shown in Figure 3.22, CRFKhuT5α cells pre-treated with arsenic for 6 hours were not more susceptible to N-MLV infection. The observed translocation and degradation of TRIM5α within cytoplasmic bodies upon arsenic treatment might be preceded by one or more postranslational modifications (phosphorylation, ubiquitination, sumoylation, acetylation) which could cause deregulation of TRIM5α and enhancement of retroviral infection. These mechanisms of modulating protein activity are much faster than degradation and might explain the ability of As_2O_3 to enhance retroviral infection when added together with the virus. The PML protein must be sumoylated prior to its degradation and a single lysine (K160) is responsible for this posttranslational modification (Lallemand-Breitenbach *et al.*, 2001). In order to find out, if TRIM5α shares the same fate as that of PML protein, we decided to mutate two sites (three lysines) within human TRIM5α that were predicted with high probability of becoming sumoylated *in vivo* (lysine at position 10 and two adjacent lysines at positions 263 and 264 of human TRIM5α). As Figure 3.24 shows, these mutants lost neither their restricting capabilities, nor their ability to respond to arsenic treatment. This, of course, does not exclude the possibility that these sites are redundant for arsenic sensitivity or that their combination with other lysines within human TRIM5α might have an effect on arsenic responsiveness. We will further continue these studies by applying the biochemistry techniques and analysing the sumoylation and phosphorylation status of

TRIM5α protein. Ultimately, studies with arsenic trioxide may help to elucidate the mechanism of TRIM5α-mediated restriction.

Chapter 4

Summary and directions for future research

In my thesis, I have described several aspects of TRIM5α-mediated restriction investigated throughout my PhD studies. Restriction factors called Ref1 in humans and Lv1 in simians, responsible for poor species-specific infectivity of certain retroviruses, were shown to be encoded by a common factor called TRIM5α. The most notable difference between the various TRIM5α orthologs was seen in their C-terminal B30.2 domain suggesting that this region of TRIM5α mostly contributes to its species-specificity and is important for virus recognition. This was supported by the examination of a short splice isoform of TRIM5α, lacking a B30.2 domain, showing its loss of restriction activity and ability to exert dominant negative effect on wild-type TRIM5α. Attempts to untangle the role of cyclophilin A in retroviral replication led to the discovery of cooperation between TRIM5α and cyclophilin A which results in a potent restriction of HIV-1 replication in simian cells. This might be explained by the prolyl-isomerisation of the HIV-1 capsid by cyclophilin A, thus making it a better target for TRIM5α. Nuclear magnetic resonance studies comparing the *cis/trans* isomerisation status of wild-type and mutant retroviral capsids could be performed to confirm this hypothesis. Investigation of the intriguing ability of arsenic trioxide to enhance retroviral replication led to the discovery of an important role for TRIM5α in this effect. The stimulation of retroviral infection by arsenic treatment seems to be due to its effects on TRIM5α deregulation and degradation. More detailed analysis of post-translational modifications of TRIM5α upon arsenic exposure will be required to fully understand its effect on this protein. Identifying the underlying biochemical pathways could lead not only to the ability to properly modulate TRIM5α activity but also to the discovery of other factors involved in TRIM5α-mediated retroviral restriction.

Despite the hard work of many laboratories, a number of interesting questions considering TRIM5α still remain unanswered. Are there any TRIM5α-binding proteins or cofactors able to influence TRIM5α activity? In such a complex and carefully controlled environment as the eukaryotic cell is it would seem improbable that TRIM5α does not have any interacting partner. Using yeast 2-hybrid, immunoprecipitation, affinity chromatography, or siRNA library studies will ultimately lead to the answer. Does TRIM5α block viruses other than retroviruses and does it have a role in cellular processes other than retroviral restriction? All viruses must appear inside the cell

cytoplasm, where TRIM5α resides. It is therefore possible that TRIM5α might directly or indirectly, through an adaptor protein, affect replication of other viral families apart from retroviruses. The capacity of diverse TRIMs to form higher-order structures, possibly recruiting multiple cellular proteins, as is the case of the PML protein, would potentially allow them to counteract cellular infection by a wide array of viruses. Of the almost 70 members of TRIM family less than 20 have been studied. Does the TRIM family represent a new class of antiviral proteins involved in innate immunity or are those few members that were shown to possess antiviral activity fortunate exceptions? A careful and comprehensive study of other TRIM members will be needed to fully elucidate the functions of this family of proteins. The large variety of proteins regulated by ubiquitination demands a high specificity for E3 ligases. This results in the growing number of E3 ubiquitin ligases and this might explain the rapid evolution of the TRIM family in metazoans. This would be a good start for their subsequent evolution into restriction factors if a virus bears recognisable capsid. An important goal for the future is thus to more precisely determine the mechanism by which TRIM5α acts and to clarify the role of ubiquitin and TRIM5's E3 ubiquitin ligase activity in this mechanism. When the mechanism of action of TRIM5α is uncovered, the next step will be to recreate its effect in a therapeutic setting.

Understanding the determinants of species-specific retroviral replication and host-virus interactions will not only enable the design of better retroviral vectors for gene therapy but also the improvement of animal models for AIDS. Because of the described TRIM5α and APOBEC3G activity HIV-1 does not replicate in most nonhuman primates. Research has therefore had to rely on animal models comprising Rhesus macaques infected with simian immunodeficiency virus (SIV_{mac}) and its derivatives. Although these models have been successful in understanding immunology and pathogenesis of AIDS disease they have limitations for testing anti-HIV therapeutics and vaccines. Recently, two studies have constructed an HIV-1 mutant viruses containing both, the entire SIV_{mac} Vif gene, and either the whole SIV_{mac} capsid or just a segment of SIV_{mac} capsid which corresponds to the cyclophilin A-binding site (Hatziioannou *et al.*, 2006; Kamada *et al.*, 2006). Introducing these changes into HIV-1 resulted in a mutant virus that is around 90% HIV-1 and is capable of spreading infection in Rhesus monkey and other simian cell lines. While the replication properties of these HIV-1 derivatives within simian cells have greatly improved *in vitro*, this success was not recapitulated *in vivo* (Hatziioannou *et al.*, personal communication) implying that additional changes will be required to resolve this issue.

There are still examples of poor permissivity to retroviral infection evident in various cell lines for which TRIM5α and other known restriction factors are not responsible. Certain HIV-1 capsid mutants (A92E, G94D) were shown to be restricted in human HeLa cells in a TRIM5α-independent and cyclophilin A-dependent way (Sokolskaja *et al.*, 2004; Hatziioannou *et al.*, 2005). The identity of the factor(s) responsible for the Lv2 phenotype, which was shown to restrict certain strains of HIV-1/2 in human cells (Schmitz *et al.*, 2004), is not known yet but is shown to be independent of TRIM5α (Kaumanns *et al.*, 2006). Apart from primates, some cell lines from rabbit (Hofmann *et al.*, 1999; Besnier *et al.*, 2002), bat or pig (Towers *et al.*, 2000; Besnier *et al.*, 2003) origin are also less permissive for certain retroviruses with TRIM5α possibly accounting for this phenotype, although this has not yet been confirmed. The importance and potency of some cellular restriction factors is supported by the fact that retroviruses, with their limited coding activity, have had to evolve special genes to counteract them. The viral infectivity factor (Vif) of HIV-1 inactivates cytoplasmic APOBEC3G, which was found to restrict HIV-1 infection in human T-lymphocytes at the stage of retroviral DNA synthesis. Another example is the viral protein U (Vpu) encoded by HIV-1 which is thought to overcome a cellular restriction responsible for the impairment of HIV-1 particle release in human cells (Varthakavi *et al.*, 2003; Neil *et al.*, 2006). The identification and characterisation of these host factors influencing viral replication is likely to impact on drug discovery and the development of animal models for viral infection and eventually, I hope, will help to combat the current HIV-1 pandemic.

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